Winter 2019

FORECASTING VIBRIO PARAHAEMOLYTICUS IN A CHANGING CLIMATE

Meghan Ann Hartwick
University of New Hampshire, Durham

FORECASTING *VIBRIO PARAHAEMOLYTICUS* IN A CHANGING CLIMATE

BY

Meghan A. Hartwick

Bachelor of Fine Arts, New York University, 2002

Masters of Conservation Medicine, Tufts University, 2012

DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

In

Molecular and Evolutionary Systems Biology

December 2019

This thesis/dissertation was examined and approved in partial fulfillment of the requirements for the degree of Ph.D. in Molecular and Evolutionary Systems Biology by:

Dissertation Director, Stephen H. Jones, Ph.D.,
Associate Research Professor, Natural Resources and the Environment,
University of New Hampshire

Cheryl A. Whistler, Ph.D.,
Professor, Molecular, Cellular and Biomedical Sciences,
University of New Hampshire

Vaughn S. Cooper, Ph. D.,
Professor, Microbiology and Molecular Genetics,
University of Pittsburgh

Jeffrey T. Foster, Ph. D.,
Associate Professor, Biological Science,
Northern Arizona University

Elena N. Naumova Ph.D.,
Professor and Chair, Division of the Nutrition Epidemiology and Data Science,
Tufts University

On November 12, 2019

Approval signatures are on file with the University of New Hampshire Graduate School.

DEDICATION

I dedicate this work to my family. No part of this would have been possible without my Mom, my Dad and my brother Mike. The strength and courage to be where I am today comes from your example.

I would also like to dedicate this to everyone who took a chance on me, made me feel like a part of this community and helped me find my place in it.

Finally, to all the guardian angels and stormy seas that kept me moving forward and out of too much trouble along the way.

ACKNOWEDGEMENTS

I have been incredibly fortunate to have an overwhelming amount of support from my friends, family, colleagues, professors and mentors every step of the way. There is no way I would have walked this far (or had this much fun) without your unending support. It should definitely be acknowledged that my first trail crew helped start this whole adventure. Later, the support of my Aunt Siobhan and Uncle Ransome opened their home and their lives to me. The crazy crew, amazing vet techs and incredibly patient veterinarians at the Marine Mammal Center and Northern Peninsula Veterinary Emergency Clinic shared their knowledge and world and helped me to find my direction.

The Tufts MCM program and especially my classmates Kelly, Christine, Jeannie, Paula, Luz, Katie, Jordan, and LaTina, brought it all together. You all continue to inspire me and I am so fortunate to count you as friends. Dr. Mark Pokras brought me back into the necropsy lab and helped me find my way to data. Almost ten years later I am so very happy to still be working with you! A very special thank you to the faculty of the Cummings School and to Dr. Gretchen Kaufman for taking a chance on me and giving me a space in this amazing program.

This work would not have been possible without my advisor, Dr. Steve Jones. Your support, time and guidance has shaped my approach to both science and life's challenges. Thank you for helping me navigate all that goes into PhD (conferences, paper writing, quals, coursework, research proposals, teaching, research, surveillance, boat maintenance and on and on). I was very green when I started, and I am so grateful for the opportunity to be part of your work. Special thanks to Kari, Randi, Audrey, Heather, Jackie, Lexi and Derek for spending all those hours in a lab or on a boat no matter the time or season. I would also like to thank NH Sea Grant, the UNH Agriculture Experiment Station, UNH School of Marine Science and Ocean Engineering, the UNH Graduate School and NH EPSCoR for funding support throughout this process.

I received a tremendous amount of support from the amazing community at the University of New Hampshire and Tufts University. Thank you to my fellow graduate students, especially Devon O'Rourke, Ben Sawicki and Sasha Kulinkina for your friendship. It has been a cornerstone and a source of sanity throughout this whole process. Special thanks to Dr. Balaji of CMC, Vellore, Dr. Michael Moore of WHOI, the students and faculty of UNDIP who have invited me into the projects and always made me feel welcome.

My committee members have gone above and beyond to incorporate me into their work, projects and labs. I was not the traditional student for Dr. Vaughn Cooper, but I can never thank you enough for the MESB program, inviting me to join your lab meetings and giving your time to help me understand the amazing work that you do. Dr. Cheryl Whistler and her entire lab have taught me so much about collaboration, teamwork and communication. I am so grateful to be involved with such important work and I can't wait to continue to contribute to exploring these research questions. Thank you so much, Dr. Jeff Foster for your insightful and important questions. They are markers I use to ground and develop my approach to explore and communicate my work. As a masters student with the MCM program, Dr. Elena Naumova once shared a whole afternoon of her time helping me with 'Meg math'. I am so grateful for every opportunity you have included me in, your amazing generosity of time, knowledge, support and the countless hours you have spent working with me since then.

The unending patience, support and love of my family got me here and kept me going. Whether it is misadventures on the high seas, world travel or sitting around the kitchen table, you have always found a way to help me stay grounded and find my strength. I am so grateful to be part of this amazing family and can't wait for the next adventure together.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

FORECASTING *VIBRIO PARAHAEMOLYTICUS* IN A CHANGING CLIMATE

BY

Meghan A. Hartwick

University of New Hampshire

The distribution, transmission and adaptation patterns of infectious diseases are changing worldwide. Though there are many potential mechanisms that can transmit infectious agents to new areas, the ability of pathogens to persist in new locations can be largely attributed to changing climate conditions, especially in temperate regions. *Vibrio parahaemolyticus,* a naturally occurring bacteria in most marine and estuarine systems, provides a model example of these globally observed climate-related changes to disease dynamics that are occurring locally in the Northeast, US. Like many *Vibrio* species, pathogenicity in human hosts is believed to be limited to a subset of strains, whereas the overall population of various strains acts as a part of the microbial community contributing to nutrient cycling and the food web. Until recently, global *V. parahaemolyticus* disease incidence was sporadic and mainly limited to the warm water regions of Asia, India and the Gulf of Mexico in the US. However, disease from pathogenic *V. parahaemolyticus* has become endemic in cold and temperate-water regions, including parts of Europe, Canada, and the Northwest and Northeast regions of the US that were historically considered low risk for *V. parahaemolyticus* disease. The consumption of raw or undercooked oysters is the most common route of *V. parahaemolyticus* infection, and the

recent increase of illnesses in the Northeast has been simultaneous with a significant expansion of the regional oyster fishery. The application of traditional environmental indicators such as water temperature and salinity that were developed in warm water regions to mitigate and manage disease risk have not been completely successful indicators for preventing the public from becoming sick due exposure to pathogenic *V. parahaemolyticus* in this region. A combination of statistical modeling and population genomic analysis was used to characterize the ecology of *V. parahaemolyticus* in the Great Bay estuary (GBE) to better inform monitoring and forecasting strategies to manage the impacts to public health and the shellfish industry of these local outbreaks, since solutions from the warm and tropical regions may not be effective in the temperate regions. Forecasting models were developed by combining ecological variables with seasonality and trend analysis to analyze long-term surveillance data collected since 2007 (Chapter 1). High resolution investigation of the interactions between *V. parahaemolyticus* and the plankton community was then used to characterize the environmental variables that contribute to the development of optimal conditions for *V. parahaemolyticus* growth over the course of a season (Chapter 2). Finally, genomic analysis of *V. parahaemolyticus* was conducted to investigate how the environment influences population structure in the GBE and may contribute to observed *V. parahaemolyticus* population dynamics (Chapter 3). Continued long-term surveillance and forecasting tools are needed to address many of the currently unresolved questions surrounding *V. parahaemolyticus* ecology that are important to better understand its role as both a member of the environmental community and an agent of human disease. This research provides an in-depth picture of the ecological drivers that underlie the interactions of *V. parahaemolyticus* with its environment and contributes to the development of effective

forecasting tools for public health and shellfish management under current and future climate scenarios.

INTRODUCTION

In recent years, disease from pathogenic *Vibrio parahaemolyticus* has emerged in cold and temperate-water regions that were historically considered low risk for *V. parahaemolyticus* disease outbreaks (Makino et al., 2003; Newton et al., 2013; CDC, 2013; Xu et al., 2015). The expansion of pathogenic strains into these regions, that were believed to be unlikely to support disease causing strains, has become a focal point of study for both public health and the seafood industry to identify the conditions that led to this new pattern of *V. parahaemolyticus* disease and prevent it from occurring in the future (Baker-Austin, Trinanes, Gonzalez-Escalona, & Martinez-Urtaza, 2017; Semenza et al., 2017; Vezzulli et al., 2013; 2009; Deter et al., 2010, McLaughlin et al., 2005).

Vibrio parahaemolyticus disease is an ongoing public health problem worldwide. Since *V. parahaemolyticus* was first identified in 1953 (Fujino et al., 1953) over fifty years of spatially and temporally intense ecological, mechanistic and genetic studies around the globe have demonstrated that *V. parahaemolyticus* is a highly adaptable organism that utilizes a complex array of mechanisms to persist in most biotic niches and abiotic conditions as a ubiquitous component of marine and estuarine ecosystems (Hartwick et al., 2019; Kaneko & Colwell, 1973; Lovell, 2017; Martinez-Urtaza et al., 2012; Turner et al., 2014; Urquhart et al., 2014.; Vezzulli et al., 2009; Jones and Summer-Brason 1998, DePaola et al., 1990). This adaptability is likely one of the main features that enables *V. parahaemolyticus* to simultaneously act as both a component of environmental ecosystems and a human pathogen (Espejo, García, & Plaza, 2017; Johnson, 2013;

Turner et al., 2016). Therefore, predicting *V. parahaemolyticus* disease risk requires a thorough characterization of its ecology.

This review is a synthesis of the ecological, virulence, and population genomic traits of *Vibrio parahaemolyticus* to address the current challenges to the development of forecasting models for *V. parahaemolyticus* disease in temperate water regions. The goal of this work is to highlight potential directions that could improve methods and produce new knowledge to address the ecological complexity of *V. parahaemolyticus* dynamics. A more in-depth understanding of these adaptation patterns will provide the foundation for the development of effective forecasting methods for *V. parahaemolyticus* risk in the temperate regions that now experience frequent and reoccurring *V. parahaemolyticus* disease outbreaks.

Ecosystem traits

The ecology of *Vibrio parahaemolyticus* has been the focus of many studies that applied a combination of long-term monitoring and intensive short-term observation across the globe. The first comprehensive report by Kaneko and Colwell (1973) detailed the complex seasonal dynamics that contributed to the emergence and persistence of *V. parahaemolyticus* in the estuarine environment of the Chesapeake Bay. It highlighted that *V. parahaemolyticus* was present in many environmental niches and that a wide range of abiotic and biotic factors are associated with its presence and accumulation in these niches, including water temperature, salinity, and plankton. Since then, the number of potentially important environmental factors have broadened considerably (Takemura, Chien, & Polz, 2014).

Abiotic

Bulk water abiotic features play a large part in the growth rate of *Vibrio parahaemolyticus* in coastal aquatic ecosystems. Temperature is recognized as the most important abiotic factor to modulate *V. parahaemolyticus* growth and concentration (Takemura, Chien & Polz, 2014). The lower threshold for growth is around 15°C, though it has been recovered from temperate and cold-water regions at 4°C (Hartwick et al., 2019; Oberbeckmann et al., 2011). The ideal range in pure cultures is between 25-35°C.

V. parahaemolyticus is halophilic and is recovered from a wide range of salinities in estuarine, marine, and brackish water, indicating that its tolerance and requirements for salinity are broad and this abiotic parameter may not be a restrictive growth parameter (Lopez-Hernandez et al., 2015; Paranjpye et al., 2015; Young et al., 2015; Oberbeckman et al., 2012, DePaola et al., 1990). Similarly, *V. parahaemolyticus* is also classified as a facultative anaerobe, indicating that dissolved oxygen (DO), which modulates inversely with water temperature, is not a restrictive growth parameter (Blackwell & Oliver, 2008; Caburlotto et al., 2010). However, in vivo studies show that ideal conditions for *V. parahaemolyticus* are a neutral pH (Wong et al., 2004). Mildly basic conditions are well tolerated, however conditions considered mildly acidic are inhibitive to growth and persistence (Wong et al., 2015). The measured ranges of environmental variables that relate to *V. parahaemolyticus* growth vary widely between studies and locations. This stems from site-specific differences in *V. parahaemolyticus* ecology. However, study design and analytic methods have also been cited as key points contributing to differences in the reported influence of these abiotic parameters (Takemura et al., 2014, Froelich and Noble, 2016).

Biotic

Studies on the biotic aspects of coastal ecosystems frequently focus on interactions between *Vibrio parahaemolyticus* and the plankton community. *V. parahaemolyticus*-plankton dynamics, first reported by Kaneko and Colwell (1975), determined that this interaction provided a key source of nutrients for growth and persistence, while also providing protection from predation and enhancing nutrient acquisition. Chitinous diatoms and dinoflagellates, such as *Skeletonemia* spp. and *Chaetocerous* spp., as well as copepods in the zooplankton community are significantly associated with the presence and concentration of *V. parahaemolyticus* in the environment (Gilbert et al., 2012). Further, chitin promotes horizontal gene transfer through chitin-induced competence, suggesting that the plankton community may play an important role in the evolutionary dynamics as well (Meibom et al., 2005; Pruzzo, Vezzulli, & Colwell, 2008).

Direct investigation of *Vibrio parahaemolyticus*-plankton dynamics can sometimes prove to be inhibitive due to the additional logistics required to effectively characterize plankton species and concentrations. As such, proxies have been applied to more effectively characterize these interactions such as chlorophyll-*a,* organic and inorganic nitrogen, phosphate, carbon, as well as silicate, (e.g. Paranjpye et al., 2015, Turner et al., 2013). Chlorophyll-*a* is frequently found to be a positively correlated parameter, whereas the statistical relationship between nutrients and *V. parahaemolyticus* are generally much more variable (Takemura, Chien and Polz, 2014, Oberbeckman et al., 2011, Blackwell and Oliver, 2008). The variable statistical relationship of nutrients to *V. parahaemolyticus* dynamics is attributed to the indirect role of nutrients in the vibrio-plankton dynamics. The importance of nutrients should not be undervalued in

characterizing the Vibrio-plankton dynamics interactions, however it is unlikely that nutrient measurements will be helpful for prediction in forecasting methods (Gude, 1985).

Different sample sources, referred to as ecosystem matrices, including sediment, water and shellfish are also a frequent interest in studies on the biotic ecology of *Vibrio parahaemolyticus* (Nilsson et al., 2019; Di et al., 2016; Deter et al., 2010). Sediment provides nutrients as well as insulation from predators for *V. parahaemolyticus*, especially during fall, winter and spring in cold and temperate water regions (Alipour et al., 2014; Vezulli et al., 2009; Kaneko and Colwell, 1973). The water column is also considered an important detection point for *V. parahaemolyticus*, though it is frequently the organic enriched fractions of the water column, including marine snow, detritus and suspended sediment that are the point of interest for these studies (Williams et al., 2014; Froelich et al., 2013). This is based on findings that suggest that *V. parahaemolyticus* prefers an attachment vs free living lifestyle and therefore is more likely to associated with these organic fractions (Johnson et al., 2012; Lovell, 2017; Parveen et al., 2008). Under certain conditions (i.e. phytoplankton blooms), *V. parahaemolyticus* can thrive in a free-living lifestyle by subsisting mainly off of polysaccharide exudate from the surrounding environment (Gilbert et al., 2012; Venkataswaran, 1990). This finding highlights the importance of algal blooms as a nutrient source for *V. parahaemolyticus*, but also provides new insight into strategies employed by free-living *V. parahaemolyticus* for persistence in the environment.

Whether shellfish are a preferred environment for *Vibrio parahaemolyticus* remains unclear, however because of their commercial importance, the shellfish-*V. parahaemolyticus* relationship is the most frequently studied interaction. The dynamics of *V. parahaemolyticus* have

been studied in many shellfish species including hard shell clams, razor clams and mussels (Lovell, 2017). However, the commercially important oyster species *Crassostrea virginica* and *C. gigas* have been the focus of the majority of *V. parahaemolyticus* studies (Zimmerman et al., 2007; DePaola et al. 1990, 2003). Filter feeding of suspended material is the most likely route by which *V. parahaemolyticus* becomes concentrated in shellfish (i.e. Froelich et al., 2013). Kaneko and Colwell (1973) first described how this filter feeding likely leads to the annual emergence of *V. parahaemolyticus* in the Chesapeake Bay as a seasonal cycle between sediment, water, plankton, and shellfish.

In addition to the major biotic relationships that have been described for *Vibrio parahaemolyticus*, incidental associations and disease in macroalgae, fish and marine mega fauna have also been reported, including outbreaks of *V. parahaemolyticus*-caused mortality in shrimp, ornamental fish and corals (Vezzulli et al. 2012), as well as isolated cases of *V. parahaemolyticus* associated abscesses and lesions in dolphins, sea otters, harbor seals, finfish, and crustaceans (Lovell, 2017; Hughes et al., 2013; Martinez-Urtaza et al., 2010).

Ecology in summary

From a public health perspective, the complex combination of conditions that relate to the growth and persistence of *Vibrio parahaemolyticus* in the environment necessitates that most preventative measures rely on the use of broad environmental indicators to prevent the public from becoming sick due exposure to *V. parahaemolyticus*. Water temperature, salinity, and *V. parahaemolyticus* concentration are among the most commonly applied criteria employed in risk assessments (DePaola et al., 2003; FDA, 2005; Lovell, 2017). However, many studies have observed that *V. parahaemolyticus* disease outbreaks from the consumption of raw or

undercooked seafood do not always coincide with high concentrations of *V. parahaemolyticus* in the environment, nor the ecosystem conditions that are thought to promote its abundance (Paranjpye et al., 2015). Ideally, preventative methods should be coupled with monitoring for conditions that enrich for potential human-specific pathogens. Such an approach is currently limited, in part, by the need to better identify the causative virulence trait in humans that would then allow determination of what conditions in the environment may promote the abundance of this trait. The penultimate virulence mechanism has not yet been confirmed though many traits associated with virulence in humans have been reported and characterized that can provide insight into the mechanisms that may contribute to *V. parahaemolyticus* disease in humans (Ceccarelli et al., 2013; Whistler et al., 2015; Xu et al., 2017, 2015).

Virulence associated traits

Vibrio parahaemolyticus disease can be caused by multiple different sequence types and recognized virulence associated traits are equally absent or present in isolates recovered from clinical patients. Given these conditions, it has been suggested that human infection and disease from *V. parahaemolyticus* may therefore be the result of trait(s) that aid in environmental persistence rather than evolved human-specific mechanism(s) like those observed in obligate or opportunistic human pathogens. For this reason, there is a growing recognition that *V. parahaemolyticus* may be an accidental human pathogen whose human pathogenicity is an exaptive trait(s) that aid in persistence in the marine and estuarine environment (Turner et al., 2017; Johnson et al., 2013).

Virulence factors in bacteria can include traits that aid in colonization, attachment, immune evasion, and competition and nutrient acquisition via toxins, all of which have been identified in *Vibrio parahaemolyticus* in the form of flagella, capsule production, hemolysins, enterotoxins, cytotoxins, proteases, siderophores and hemagglutinin (Johnson, 2013). The most recognized and studied of these traits are the hemolysins *tdh* and *trh*, though genes for the Type III Secretion Systems (T3SS) are increasingly being recognized for their potential contribution to causing disease. The virulence-associated traits tend to co-occur on pathogenicity islands (VPaI) that have are dynamically shared between strains via horizontal gene transfer and recombination. In conjunction with the inconsistent detection of *tdh*, *trh* and T3SS in clinical cases (Lovell, 2017; Nishibuchi et al., 1992; Shinoda & Miyoshi, 2006), the dynamic exchange of VPaIs and the ubiquitous presence of virulence associated traits in 'environmental' and 'clinical' strains alike have been a major hurdle to developing targeted preventative public health measures (Ceccarrelli et al., 2013).

Hemolysins

Pore forming toxins such as hemolysins are the mechanism of pathogenicity employed by many bacterial species including *Escherichia coli*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* and act by disrupting host cell membranes to directly kill target cells, to evade immune detection, and/or to release nutrients (Los et al., 2013). Hemolysin gene products of *Vibrios* have been shown to lyse host erythrocytes and may be used to access the nutrients bound within host cells. The thermolabile hemolysin gene (*tlh*), thermostable direct hemolysin (*tdh*) and the *tdh*-related hemolysin (*trh*) genes are the most commonly used potential virulence traits in *Vibrio parahaemolyticus* (Xu et al., 2017; Lovell, 2017; Ceccarelli et al., 2013;

Johnson et al., 2013). They are generally used in a multiplex polymerase chain reaction to identify *V. parahaemolyticus* and help to differentiate potential pathogenic strains. The first PCR primers developed for detection of *tlh*, *tdh*, and *trh* were described by Bej et al. (1999) who used them in multiplex PCR to detect all three genes simultaneously (Lovell, 2017).

The *tlh* gene encodes a thermolabile hemolysin. The specific function of this gene in human infection is unknown though the *tlh* gene is widely considered to be a species-specific marker for *Vibrio parahaemolyticus* (Klein et al., 2014; Johnson, 2013). The *tdh* and *trh* genes, are approximately 67% identical and are predicted to function in similar manners (Johnson et al., 2013). Products of *tdh*, an amyloid toxin and *trh*, which is believed to act by activating Cl- channels (Ceccarelli et al., 2013), embed in and disrupt host cell membranes, acting as porins. This can be detected in vitro by lysis of erythrocytes, as demonstrated by β-hemolysis (the Kanagawa phenomenon) on saline blood agar (Wagatsuma Agar)(Klein et al., 2014; Lovell, 2017). Both *tdh* and *trh* sequences can vary widely, they are separated on the two chromosomes and are typically harbored on islands (Xu et al., 2017) in many *V. parahaemolyticus* strains (Lovell, 2017).

In 1996, the surveillance testing in Kolkata, India, determined that a novel serotype, O3:K6, accounted for 50–80% of *Vibrio parahaemolyticus* gastroenteritis infections were *tdh*+/*trh*- (Ceccarelli., et al., 2013). Whereas almost all *V. parahaemolyticus* strains isolated from clinical samples possess beta-hemolytic activity attributed to these two genes (Ceccarelli et al., 2013), about 10% of clinical strains do not contain *tdh* and/or *trh* (Xu et al., 2015; Raghunath, 2014). More in-depth environmental studies have now shown that the detection of *tdh* and *trh* in the environment can vary from to 1–2% of total strains to upwards 48-52% of isolates. Whereas *tdh*

and *trh* are still used to identify potential virulent strains, the prevalence of these genes in environmental *V. parahaemolyticus* strains has led many to suspect that their role is not solely to contribute to human disease (Lovell, 2017).

Secretion systems

The range of hemolysin profiles in *Vibrio parahaemolyticus* strains recovered from patients with clinical *V. parahaemolyticus* disease prompted many researchers to look beyond *tdh, trh* and *tlh* to determine additional factors that may underlie virulence in *V. parahaemolyticus.* Secretion systems, which are used by most bacterial species for routine functionality can be essential to pathogenesis for *Salmonella, Shigella* and *Yersinia* (Hapfelmeier et al., 2005). Six secretion systems (T1SS-T6SS) have been described in gram negative bacteria, of which two (T3SS and T6SS) are of central interest as potential sources of virulence in *V. parahaemolyticus* because of their recognized role in promoting toxicity, immune evasion and cell adherence (i.e. Zhang & Orth, 2013).

T3SS use a 'needle-like apparatus' to insert a range of effector proteins that can cause cytotoxicity or enterotoxicity (Ceccarelli et al., 2013) or inhibit immune systems or forcing the induction of host cell apoptosis (Blondel et al., 2016; Zhang & Orth, 2013). They are also important for attachment and colonization in intestinal systems and in extra-intestinal systems. T3SS1, identified by Makino et al., (2003) is located on chromosome one and is well conserved and widespread in both clinical and environmental strains of *V. parahaemolyticus* (Ceccarelli et al., 2013). Identified as *vscC1*, the T3SS1 gene cluster is composed of 42 genes (Lovell, 2017). Collectively, T3SS1 effectors are reported to evade the host immune response and to cause

cytotoxic damage in the host cells to acquire nutrients from non-erythrocyte host cells Johnson (2013).

T3SS2 is found on chromosome two, identified by *vscC2* (Makino et al. 2003) and there are two T3SS2 variants (Johnson, 2013; Lovell, 2017). T3SS2 is believed to be necessary to deliver toxin proteins into host cells and plays a role colonization, immune avoidance, and acquisition of nutrients. Unlike T3SS1, it is not present in all *Vibrio parahaemolyticus* strains. Because of its variability and dual role in toxicity and immune avoidance, the gene for an outer membrane protein (*vsc*C) has also been used as a marker for virulent *V. parahaemolyticus* (Klein et al., 2014; Park et al., 2004). Recent findings demonstrated that, along with its six effectors, T3SS2- α allows *V. parahaemolyticus* to invade, survive, and replicate in non-phagocytic host cells (Zhang & Orth, 2013).

Two T6SS have also been identified in *Vibrio parahaemolyticus* and are distributed between the two chromosomes (Boyd et al., 2008). T6SS were only recently identified by (Pukatzki et al., 2006) in *V. cholerae*, where it is believed to contribute to mediate extracellular export of virulence factors and injection into eukaryotic host cells (Boyd et al., 2008). Its role in *V. parahaemolyticus* has not yet been fully determined (Johnson, 2013). Preliminary data suggest that it is involved in adhesion to host cells (Yu et al., 2012). Since T6SS2 and T3SS2 co-exist, it was proposed that the two systems might cooperate during infection. T6SS2 plays its role in adhesion, the first step of infection, and T3SS2 exports effectors by inducing entero-cytotoxicity (Ceccarelli et al., 2013; Yu et al., 2012; Park et al., 2004)

Interactions between virulence factors have been suggested not only within secretion systems but also between hemolysins and secretion systems. Early studies, which reported a

correlation between the presence of T3SS2α and *tdh* and separately T3SS2β with *trh*, led to the hypothesis that the former was a requirement for strains to be Kanagawa positive (Baker-Austin et al., 2010). Whereas recent findings appear to disprove this correlation based on the detection of a wide range of combinations of *tdh*, *trh* and T3SS2s and Kanagawa phenotype within strains (Jones et al., 2012; Paranjpye et al., 2012), it has been determined that hemolysins and secretion systems tend to co-occur in regions of the genome referred to as pathogenicity islands (Lovell, 2017; Xu et al., 2017; Klein et al. 2014; Ceccarelli et al., 2013)

Vibrio pathogenicity islands

Pathogenicity islands are groups of genes with virulence-associated traits that can be transferred and acquired holistically by horizontal gene transfer facilitated by phage, plasmid or induced competence. They carry genes that can provide some benefit to *V. parahaemolyticus* for persistence, usually to enhance competition or nutrient acquisition (Johnson, 2013; Ceccarelli et al., 2013) Nine pathogenicity islands have been defined in RIMD (VPaI-1 to VPaI-9), and these can be located on either chromosome and are differentially distributed between strains (Boyd et al., 2008; Hurley et al., 2006). Homologous VPaIs have been identified in other strains. Initially, they were considered a useful marker to identify specific pathogenic strain types, particularly those that were associated with pandemic disease. However, it has been difficult to establish a pattern between the presence of a VPaI and strains that could be classified as a pandemic, environmental or clinically associated. Strains of sequence-types or serotypes that are associated with disease do not always contain the VPaI to which they are generally attributed, and non-pathogenic non-pandemic strains have been found to contain the VPaIs typically used as markers for pathogenic strains (Makino et al., 2003).

Despite the rapidity of exchange that appears to occur with VPaIs between strains, Xu et al., (2017) recently demonstrated that they can be invaluable to characterizing early events that lead to the differentiation of environmental strains to become potential human pathogens. Further, the variable presence and content of VPaIs provides enormously valuable insight into the amount and frequency of exchange of genetic materials that occurs within the *V. parahaemolyticus* genome in the environment. The size and GC content of these VPaIs suggests that they are most frequently acquired via horizontal gene transfer (Boyd et al., 2008; Hurley et al., 2006). In addition, the genetic content of these islands varies between genetic material that is shared between *V. parahaemolyticus* strains and material that may have been acquired from other species, for example: homologs of the *Escherichia coli* cytotoxic necrotizing factor (CNF) and *Pseudomonas* exoenzyme T identified in VPaI-7 (Ceccarelli et al., 2013; Makino et al., 2003)

Pathogenicity islands show potential utility as a marker that can be used to identify and differentiate strains with pathogenic potential as well as a tool to observe the dynamics of genetic exchange via constant recombination within *V. parahaemolyticus*. However, in addition to hemolysins and T3SS and T6SS, *V. parahaemolyticus* is also equipped with an additional suite of genetic traits that are considered virulence-associated traits. These include quorum sensing, biofilm formation, proteases and chitinases, and siderophores (Johnson, 2013).

Quorum sensing and biofilms

Quorum sensing is used by many bacterial species to regulate cell density through chemical signaling to control gene expression (Johnson, 2013). This process of signaling has been shown to regulate hundreds of genes involved in virulence factor production and growth. *AphA* and *OpaR* are the two master regulators of quorum sensing in *Vibrio parahaemolyticus* (Sun

et al., 2012; Zhang et al., 2012), where *AphA* is expressed at low cell density and *OpaR* is expressed at high cell density (Zhang et al., 2017). Expression of T3SS secretion machinery genes in *V. parahaemolyticus* (T3SS1) are dependent upon a functional quorum sensing system; at high cell densities, quorum sensing decreased T3SS activity in both species (Johnson, 2013). Recent work suggests that quorum sensing plays a role in the self-limiting dynamics of gastro-intestinal infection by *V. parahaemolyticus* and may have an ecological advantage for resource competition.

In addition to controlling the production of metabolites, quorum sensing also regulates the production of biofilms (Davey & O'toole, 2000; Jayaraman & Wood, 2008). Biofilm formation is a complex process that involves the production of a polysaccharide matrix that acts as a mechanism of attachment and protection from external threats from the host and other bacterial species. It has been shown to be essential for colonization of a host and in vitro studies have demonstrated that strains that are deficient in biofilm production factors are less successful in causing infection and disease (Johnson, 2013).

Chitinases and proteases

Chitin is one of the most abundant molecules in the marine and estuarine environment and is used by *Vibrio parahaemolyticus* as a source of nutrients. *V. parahaemolyticus* produces chitinases, a class of enzymes that can breakdown chitin into accessible carbon monomers. *GbpA*, one of the more well characterized genes involved in this process has also been shown in *V. cholerae* to facilitate attachment to surfaces as well including chitinaceous plankton and intestinal cell wells (Johnson, 2013). In vitro studies show that mutants lacking *GbpA* had lowered resilience to unfavorable environmental conditions including various temperatures

and salinities (Johnson, 2013). The study of the function of *GbpA* in *V. parahaemolyticus* is ongoing (Tiruvayipati & Bhassu, 2016; Tiruvayipati et al., 2013) but preliminary outcomes from in vivo observation suggest its serves a similar role in attachment and chitin utilization.

Proteases are another class of enzymes used by *Vibrio parahaemolyticus* to access nutrients. Target-specific proteases convert proteins into usable forms for *V. parahaemolyticus*. Proteases are key toxicity factors in many pathogenic bacterial species for example *Bacillus anthracis*, the causative agent of anthrax and are recognized to play pathogenic roles following the initial infection (Shimodo and Myoshi, 2006). Two metalloproteases and one serine protease has been identified in *V. parahaemolyticus*. The serine protease, encoded by *proA* has been shown to cause erythrocyte lysis, cell toxicity and death in mice and cell culture (Johnson, 2013). The metalloproteases in *V. parahaemolyticus* are encoded by *PrtV* and *VppC*. Whereas metalloproteases have been shown to be key components of both botulinum and tetanus toxin, their potential role in *V. parahaemolyticus* has not been well characterized (Shimodo and Myoshi, 2006).

Virulence associated traits in summary

A thorough assessment of the virulence-associated traits found in *Vibrio parahaemolyticus* provides insight into the potential mechanisms it employs for persistence in the environment and the human host. The virulence-associated traits identified in *V. parahaemolyticus* can be considered as two components of the disease process. Traits such as hemolysins (*tdh* and *trh)*, toxins from secretion systems and proteases produce the diseased state, whereas traits involved in motility (flagella), immune evasion (capsule production) and adhesion (biofilm formation)

enable *V. parahaemolyticus* to overcome host defenses or competition to establish the infection (Lee et al. 2015; Ceccarelli et al. 2013; Shinoda and Miyoshi, 2006).

The lack of a definitive human disease-causing trait that all pathogens contain is clearly one of the most important challenges to overcome in order to develop targeted methods for forecasting disease risk. In its absence, virulence associated markers or traits such as *tdh*, *trh*, and more recently T3SS and VPaIs have been used as markers to differentiate potential pathogens from total *Vibrio parahaemolyticus* to provide some differentiation of risk between inert and potential pathogenic strains, but these approaches have noted limitations. Successful forecasting methods for *V. cholerae* have focused on O1 and O139, and likewise monitoring for *V. parahaemolyticus* disease risk have focused on detection of genes in known pathogenic serotypes or sequence types such as ST3 and ST36, which are known to have pandemic distribution. However, because of the rapidity of genetic exchange between strains, this has been found to have limitations as well. In part because the pandemic strain has evolved since its emergence in 1998 and isolates have been found to be missing key virulence associated traits. Further, environmental *V. parahaemolyticus* strains have been isolated with pathogenicity islands identified to be key to human virulence including VPaI-7 andVPaI-2 (Ceccarelli et al., 2013; Gennari et al., 2012). Given this, effective epidemiological reconstruction and investigation of the mechanisms of pathogen emergence will require accounting for the many potential sources of underlying diversity within populations by accounting for the evolutionary dynamics acting on *V. parahaemolyticus* at both the genome and population level. These particular features make epidemiological phylogenetic analysis and risk forecasting of *V. parahaemolyticus* disease especially challenging.

Population genomics and genetics

Rapid development of low cost, high throughput sequencing in recent years has provided tremendous insight into the evolutionary and population dynamics of *Vibrio parahaemolyticus* that contribute to the genetic flexibility that is a characteristic trait of *V. parahaemolyticus*. The complete genome of pandemic strain RIMD, published in 2003 (Makino et al. 2003), provided the first real opportunity to assess the basic structure and functionality of *V. parahaemolyticus* in its entirety. The first complete representative genome of *V. parahaemolyticus* was a pandemic O3:K6 strain, now commonly referred to as RIMD (2210633) that was recovered from an individual with food poisoning in 1996. RIMD is composed of two circular chromosomes, containing approximately 4832 coding sequences, with a GC bias of 45.4% and approximately 40% of coding sequences were annotated as hypothetical proteins. Chromosome one was found to be larger, containing 3080 coding sequences, many of which were involved in basic cell functions such as growth and viability, whereas chromosome two consisted of approximately 1752 coding sequences and had more genes related to metabolism and environmental regulation.

Since it was first sequenced, over 800 *Vibrio parahaemolyticus* genome assemblies have been made available through NCBI and >1800 ST profiles are included in the *V. parahaemolyticus* multi locus sequence type (pubMLST) database. This helps to illustrate why forecasting disease risk has proven such a challenge by demonstrating the amount of diversity that evolves and is maintained in *V. parahaemolyticus* populations. The variable presence and mobility of virulence-associated traits can be attributed to the major forces of mutation, horizontal gene transfer and recombination shaping the *V. parahaemolyticus* genome. Whereas both variable mutation rates

across the genome and horizontal gene transfer and have been shown to play a role in *V. parahaemolyticus* evolution (Tamames, Sánchez, Nikel, & Pedrós-Alió, 2016), many believe that HGT and recombination is the predominant force shaping the *V. parahaemolyticus* genome (Ceccarelli et al., 2013; Johnson, 2013; Martinez-Urtaza et al., 2017). Historically, phylogenetic assessment has been the most widely used epidemiological tool to trace the evolutionary background or trajectory of the emergence of virulence within a population. However, the ability of *V. parahaemolyticus* to undergo recombination, even in conserved regions believed to be limited to vertical acquisition, potentially masks many of the patterns typically used to assess the development and succession of virulent lineages within largely non-pathogenic populations. Given this, effective epidemiological reconstruction and investigation of the mechanisms of pathogen evolution will require accounting for the many potential sources of underlying diversity within populations.

Recombination and mutation

The structure and content of the *Vibrio parahaemolyticus* genome appears to be largely facilitated by variable mutation rates combined with gene gain and loss. The overall genomic content consists mostly of coding sequences (identified and hypothetical) with very little intragenic or pseudogenic content (~5,000 orf and 5mb). This is consistent with what is observed in most free-living bacterial species where gene number is observed to be a direct linear relationship to genome size.

The process that results in this densely packed genome is known as genome reduction. A number of factors can contribute to this, each providing insight into the evolutionary forces shaping the genome. Metabolic efficiency has been suggested as one these selective

mechanisms. *Vibrio parahaemolyticus,* which has 11 copies of rRNA operons, higher than in many other prokaryotes (Makino et al., 2003), can undergo replication in ideal environmental conditions every 8 minutes. The metabolic cost of maintaining non-essential genetic material could be a prohibitive cost in resource limited environments (Abu Kwaik & Bumann, 2013, 2015). The genome streamlining hypothesis, which has mixed support from experimental and comparative studies, proposes that material that is not essential represents a negative metabolic cost and therefore selection deletes superfluous content from the genome via mutation accumulation and deletion (Bobay & Ochman, 2017; Weinert & Welch, 2017). Therefore, genome reduction would make the organism more fit for rapid replication in nutrient-limited environments. In contrast, genome reduction in host-associated pathogens occurs through gradual loss of function in regions that are no longer needed by the organisms and are recognizable by the presence of extra genetic material including pseudogenes (Bobay and Ochman, 2017).

Genome reduction has also been proposed to indicate environmental adaptation and evolution within the individual towards niche specialization. Martinez-Urtaza et al., (2017), found a trend toward gene number reduction in ST36, where larger genomes and higher gene numbers were observed in strains from older subpopulations than in modern U.S. strains, suggesting that the gene number reduction could be associated with a more specialized lifestyle as a result of niche adaptation in the US. Thus, genome reduction may have multiple evolutionary benefits to control the metabolic cost to *Vibrio parahaemolyticus* and facilitate adaptation when exposed to new selective pressures (Bobay & Ochman, 2017).

The major driver of evolution and adaptation in *Vibrio parahaemolyticus*, however, is attributed to the acquisition of novel genetic material through homologous and non-homologous recombination and mutation (Bobay and Ochman, 2017, Ceccarelli et al., 2013; Johnson, 2013). Horizontal gene transfer through recombination, phage or plasmid enables bacteria to adapt to new environmental niches, trait sharing such as antibiotic resistance or pathogenicity islands (Metzger & Blokesch, 2014). Homologous recombination also provides the resources for niche adaptation and diversification. The current genetic divergence within ST36 clonal populations has been attributed to recombination (Martinez-Urtuza et al., 2017, Xu et al., 2017). Whereas the most recognized mechanism for rapid niche adaptation in *V. parahaemolyticus* is homologous recombination and horizontal gene transfer, Johnson et al., (2013) suggested that elevated mutation rates may also facilitate niche adaptation.

The diversity among clinical and environmental *Vibrio parahaemolyticus* populations has frequently been examined using multilocus sequence analysis and these studies reveal highly diverse populations. Many hypothesize that organizing the diversity of *V. parahaemolyticus* to environmentally relevant structures within these populations could be key to understanding and forecasting *V. parahaemolyticus* dynamics and disease risk (Fraser et al., 2009). However, assigning this diversity to ecological drivers has proven to be complex. In one study, diversity was associated with geography (Cui et al., 2015), however most traditional phylogenetic methods such as Multi-Locus Sequence Typing (MLST) have not produced isolate clustering that relates to geographic, environmental, or pathogenicity factors (Ellis et al., 2012; Thompson et al., 2004; Urmersbach et al., 2014).

Ecotypes

The diversity within and between *Vibrio parahaemolyticus* populations can be driven by the demands of the environment they inhabit. This idea of "niche sequestering" was observed and reported by Johnson et al., (2012) in strains from the Gulf of Mexico and Shapiro and Polz (2014) where *V. parahaemolyticus* strains from similar environmental niches were more similar in genetic content and allelic diversity than they were to strains that were isolated from different environmental conditions. These observations are part of the basis for proposing that *V. parahaemolyticus* populations may be structured into ecological species, or ecotypes. Ecotypes can be understood as sub-populations that interact with other strains within their niche but have limited exposure to *V. parahaemolyticus* that do not interact within the same microhabitats in the environment. Because of this genetic isolation, strains that inhabit similar niches will have more similar allelic diversity and accessary genomes (Friedman, Alm, & Shapiro, 2013).

Ecotypes provide an appealing mechanistic concept to connect environmental conditions to population dynamics, and the water column provides a diverse range of resources and nutrient patchiness that is an ideal environment for the development of micro-niche specialization (Cohan, 2002; Cordero & Polz, 2014; Hunt et al., 2008; Shapiro & Polz, 2014). Though Keymer et al., (2007) and Hunt et al., (2008) have identified potential *Vibrio* ecotypes by allelic variation or variable genome content, ecotypes have not been frequently identified within environmental populations (Bendall et al., 2016).

2nd Chromosome adaptation

Vibrio parahaemolyticus may also more rapidly undergo environmental adaptation through its smaller, second chromosome. The second chromosome in *Vibrio* is hypothesized to

have arisen from the acquisition of a plasmid that provided a fitness benefit and was maintained and evolved to be essential to *V. parahaemolyticus* function. It has been noted frequently that the second chromosome tends to be enriched in genetic functions related to environmental persistence, leading to the theory that the second chromosome retains its role as a resource for the integration of novel material for environmental adaptation and persistence (Ellis et al., 2012; Makino et al., 2003; Morrow & Cooper, 2012). In other organisms, this concept is supported by higher substitution rates, greater dispensability, and lower codon usage bias for genes on secondary chromosomes (Cooper et al., 2010; Holden et al., 2004). Similarly, GC bias and a higher proportion of genes unique to each isolate (chromosome one: 56.8%, chromosome two: 29.5%) have been reported in *V. parahaemolyticus* (Ceccarelli et al., 2013, Makino et al., 2003).

Population dynamics in summary

The complexity of predicting *Vibrio parahaemolyticus* dynamics can be better understood through the scope of the mechanisms that drive its genomic and genetic diversity. SNP analysis of regions of interest, most frequently within housekeeping genes, has been used extensively to establish genetic relationships between strains and potentially trace the emergence of pathogenic lineages. However, horizontal gene transfer through homologous and non-homologous recombination is also a major driving force of *V. parahaemolyticus* evolution that may provide more information about the ecological drivers that are shaping the population (Metzger and Blokesch, 2014). Therefore, the population genetics and genomics are shaped by both lineage and the environment (Tamames et al., 2016). The relationship between gene

content and the environment remains to be clarified, however the mechanisms potentially contributing to ecotype differentiation and niche specific adaptation through variable genome content are an important potential direction to better determining the basis for how environmental adaptation shapes *V. parahaemolyticus* dynamics.

Forecasting disease risk

Since the link between *Vibrio* disease dynamics and the environment was first recognized, there has been a huge effort worldwide to characterize the ecology of *Vibrio parahaemolyticus* to understand where and when human-health risks will occur (Nilsson et al., 2019; Vezzulli et al., 2009). Takemura, Chien and Polz (2014) provided an excellent summary of the differing and often conflicting outcomes of the reported correlative relationships between *V. parahaemolyticus* and the more common environmental variables that are assessed including water temperature, salinity, dissolved oxygen, turbidity and chlorophyll-*a*. This ecological complexity could arise from region-specific and even site-specific environmental differences as well as differences in local *Vibrio parahaemolyticus* population genetics and genomics (Froelich and Noble, 2016; Shapiro and Polz, 2014). These are certainly contributing factors, however there are also widely ranging differences in analytic, temporal and spatial study designs that may also be contributing to this observed complexity. For example, though multiple regression and correlation analysis are the most frequently applied analytic methods, season-specific segmentation, lagged relationships exceeding one month, polynomial transformations and descriptive splines between ecological and temporal relationships (Nilsson et al., 2019; Davis et al., 2019; 2017; Paranjpye et al., 2015; Froelich et al., 2012) are also used and could influence the importance of observed relationship of environmental variables. The majority of studies are also

often short-term observations between one summer season or less than two years, and frequently group together multiple monitoring stations that may have widely ranging site characteristics. Though ecological inference may be made from these kinds of studies, they are limited in their ability to provide standardized comparisons between regions or to develop transferable forecasting models.

Other important considerations within these outcomes of *Vibrio parahaemolyticus* ecological studies are geographic distribution, transmission and adaptation patterns. *V. parahaemolyticus* concentrations are highly seasonal in the Northeast, US where the dominate seasonal driver is temperature, and likewise, *V. parahaemolyticus* dynamics are most strongly correlated with water temperature. In other regions, where *V. parahaemolyticus* dynamics are also seasonal but the environmental driver of seasonality is monsoon- driven rainfall, the relationship between temperature and *V. parahaemolyticus* is not as prominent (Deepanjali et al., 2005). Clearly this does not mean that *V. parahaemolyticus* dynamics in tropical regions would not biologically respond to water temperature variation. Rather it relates to the covarying variability of the environmental variable with *V. parahaemolyticus* and the statistical relationship that would be observed.

Seasonality, where regular and predictable changes in environmental and climatic conditions re-occur every calendar year, tends to become more pronounced with increasing distance from the equator and is largely due to extreme temperature variation driven by variable day-length (Tonkin et al., 2017). *Vibrio parahaemolyticus* concentrations in the Great Bay estuary (GBE) are highly seasonal and follow the same pattern each year that mirror water temperature. Concentrations increase rapidly each springtime as water temperatures increase,

and peak around the warmest summer conditions then decrease as water temperatures decrease in the fall each year. This water temperature-driven seasonality is also strongly intercorrelated with most other environmental variables. So, though a complex combination of environmental variables likely influences *V. parahaemolyticus* dynamics, the strength of the correlative relationship between *V. parahaemolyticus* and water temperature and collinearity with other environmental variables in temperate regions obscures the contribution of other variables that may also be important to effective ecological and forecasting models. Modeling approaches such as harmonic regression that incorporate this seasonality could provide the structure to overcome these challenges and provide the basis to untangle the complexity of the environmental variables contributing to *V. parahaemolyticus* dynamics in the GBE.

The recent increase in shellfish-borne illnesses in the Northeast US has resulted in application of intensive management practices based on a limited understanding of when and where risks are present. Temperature and salinity are cited as the most influential environmental variables for *Vibrio parahaemolyticus* dynamics. However, the application of these variables in risk management has had limited efficacy in cold and temperate water regions where *V. parahaemolyticus* disease has become an established public health issue. This work is a targeted investigation into the ecology and population genetics of *V. parahaemolyticus* using the GBE long-term surveillance data. A combination of statistical modeling and population genomic analysis was used develop forecasting models (Chapter 1) provide a high resolution analysis of the interactions between *V. parahaemolyticus* and the plankton community (Chapter 2), and genomic analysis of *V. parahaemolyticus* to investigate how the influences population structure in the GBE (Chapter 3). This research provides a more in-depth picture of the drivers

that underlie the interactions of *V. parahaemolyticus* with its environment and contributes to the development of effective forecasting tools for public health and shellfish management under current and future climate scenarios.

References

Abu Kwaik, Y., & Bumann, D. (2013). Microbial quest for food *in vivo* : 'Nutritional virulence' as an emerging paradigm. *Cellular Microbiology*, *15*(6), 882–890. https://doi.org/10.1111/cmi.12138

Abu Kwaik, Y., & Bumann, D. (2015, June 1). Host Delivery of Favorite Meals for Intracellular Pathogens. *PLoS Pathogens*. Public Library of Science. https://doi.org/10.1371/journal.ppat.1004866

Alipour, M., Issazadeh, K., & Soleimani, J. (2014). Isolation and identification of *Vibrio parahaemolyticus* from seawater and sediment samples in the southern coast of the Caspian Sea. *Comparative clinical pathology*, 23(1), 129-133.

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., & Martinez-Urtaza, J. (2017). Non-Cholera Vibrios: The Microbial Barometer of Climate Change. *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2016.09.008

Bendall, M. L., Stevens, S. L. R., Chan, L. K., Malfatti, S., Schwientek, P., Tremblay, J., … Malmstrom, R. R. (2016). Genome-wide selective sweeps and gene-specific sweeps in natural bacterial populations. *ISME Journal*, *10*(7), 1589–1601. https://doi.org/10.1038/ismej.2015.241

Blackwell, K. D., & Oliver, J. D. (2008). The ecology of *Vibrio vulnificus, Vibrio cholerae,* and *Vibrio parahaemolyticus* in North Carolina estuaries. *Journal of Microbiology (Seoul, Korea)*, *46*(2), 146–153. https://doi.org/10.1007/s12275-007-0216-2

Blondel, Carlos J., Joseph S. Park, Troy P. Hubbard, Alline R. Pacheco, Carole J. Kuehl, Michael J. Walsh, Brigid M. Davis, Benjamin E. Gewurz, John G. Doench, and Matthew K. Waldor. (2016). CRISPR/Cas9 screens reveal requirements for host cell sulfation and fucosylation in bacterial type III secretion system-mediated cytotoxicity. Cell host & microbe 20, no. 2: 226-237.

Bobay, L.-M., & Ochman, H. (2017). Biological Species Are Universal across Life's Domains. *Genome Biology and Evolution*, *9*(3), 491–501. https://doi.org/10.1093/gbe/evx026

Boyd, E. F., Cohen, A., Naughton, L. M., Ussery, D. W., Binnewies, T. T., Stine, O. C., & Parent, M. A. (2008). Molecular analysis of the emergence of pandemic *Vibrio parahaemolyticus*. *BMC Microbiology*. https://doi.org/10.1186/1471-2180-8-110

Caburlotto, G., Haley, B. J., Lleò, M. M., Huq, A., & Colwell, R. R. (2010). Serodiversity and ecological distribution of *Vibrio parahaemolyticus* in the Venetian Lagoon, Northeast Italy. *Environmental Microbiology Reports*, *2*(1), 151–157. https://doi.org/10.1111/j.1758-2229.2009.00123.x

Ceccarelli, D., Hasan, N. A., Huq, A., & Colwell, R. R. (2013). Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Frontiers in Cellular and*

Infection Microbiology. https://doi.org/10.3389/fcimb.2013.00097

Centers for Disease Control and Prevention (CDC). (2013). Increase in *Vibrio parahaemolyticus* illnesses associated with consumption of shellfish from several Atlantic coast harvest areas, United States, 2013. Vibrio Illness (Vibriosis), 21.

Cohan, F. M. (2002). What are Bacterial Species? *Annual Review of Microbiology*, *56*(1), 457–487. https://doi.org/10.1146/annurev.micro.56.012302.160634

Cooper, V. S., Vohr, S. H., Wrocklage, S. C., & Hatcher, P. J. (2010). Why genes evolve faster on secondary chromosomes in bacteria. *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1000732

Cordero, O. X., & Polz, M. F. (2014). Explaining microbial genomic diversity in light of evolutionary ecology. *Nature Reviews Microbiology*. Nature Publishing Group. https://doi.org/10.1038/nrmicro3218

Davey, M. E., & O'toole, G. A. (2000). Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/MMBR.64.4.847-867.2000

Davis, B. J., Jacobs, J. M., Davis, M. F., Schwab, K. J., DePaola, A., & Curriero, F. C. (2017). Environmental determinants of *Vibrio parahaemolyticus* in the Chesapeake Bay. Appl. Environ. Microbiol., 83(21), e01147-17.

Davis, B. J., Jacobs, J. M., Zaitchik, B., DePaola, A., & Curriero, F. C. (2019). *Vibrio parahaemolyticus* in the Chesapeake Bay: operational in situ prediction and forecast models can benefit from inclusion of lagged water quality measurements. Applied and environmental microbiology, 85(17), e01007-19.

Deter, J., Solen, L., Antoine, V., Jaufrey, J., Annick, D., Dominique, H.H. (2010). Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. Environmental microbiology, 12(4), 929-937.

Deepanjali, A., Kumar, H., Karunasagar, I., Karunasagar, I. Seasonal Variation in Abundance of Total and Pathogenic *Vibrio parahaemolyticus* in Oysters along the Southwest Coast of India. Applied and Environmental Microbiology. (2005) 71, 7. http://aem.asm.org/content/71/7/3575.abstract

DePaola, A., Ulaszek, J., Kaysner, C. A., Tenge, B. J., Nordstrom, J. L., Wells, J., Gendel, S. M. (2003). Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.69.7.3999-4005.2003

DePaola, A.; Hopkins, L.H.; Peeler, J.T.; Wentz, B.; McPhearson, R.M. Incidence of *Vibrio parahaemolyticus* in US coastal waters and oysters. Appl. Environ. Microbiol. 1990, 1;56(8), 2299-302.

Ellis, C. N., Schuster, B. M., Striplin, M. J., Jones, S. H., Whistler, C. A., & Cooper, V. S. (2012).

Influence of seasonality on the genetic diversity of *Vibrio parahaemolyticus* in new hampshire shellfish waters as determined by multilocus sequence analysis. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.07794-11

Espejo, R. T., García, K., & Plaza, N. (2017, July 24). Insight into the origin and evolution of the *Vibrio parahaemolyticus* pandemic strain. *Frontiers in Microbiology*. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2017.01397

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G., & Hanage, W. P. (2009). The Bacterial Species Challenge : Ecological Diversity. *Science*, *323*(February), 741–746. https://doi.org/10.1126/science.1159388

Friedman, J., Alm, E. J., & Shapiro, B. J. (2013). Sympatric Speciation: When Is It Possible in Bacteria? *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0053539

Froelich, B., Bowen, J., Gonzalez, R., Snedeker, A., & Noble, R. (2013). Mechanistic and statistical models of total vibrio abundance in the neuse river estuary. *Water Research*. https://doi.org/10.1016/j.watres.2013.06.050

Froelich, B. A., & Noble, R. T. (2016). *Vibrio* bacteria in raw oysters: managing risks to human health. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371(1689), 20150209.

Fujino, T., Okuno, Y., Nakada, D., Aoyama, A., Fukai, K., Mukai, T., & Ueho, T. (1953). On the bacteriological examination of shirasu-food poisoning. *Medical Journal of Osaka* University, 4(2/3), 299-304.

Gennari, M., Ghidini, V., Caburlotto, G., & Lleo, M. M. (2012). Virulence genes and pathogenicity islands in environmental Vibrio strains nonpathogenic to humans. *FEMS Microbiology Ecology*, *82*(3), 563–573. https://doi.org/10.1111/j.1574-6941.2012.01427.x

Gilbert, J. A., Steele, J. A., Caporaso, J. G., Steinbrück, L., Reeder, J., Temperton, B., Field, D. (2012). Defining seasonal marine microbial community dynamics. *The ISME Journal*. https://doi.org/10.1038/ismej.2011.107

Hartwick, M., Urquhart, A.E., Whistler, C.A., Cooper, V.S., Naumova, E.N., and Jones, S.H. Forecasting *Vibrio parahaemolyticus* Concentration in Shellfish from a New England Estuary (submitted)

Holden, M. T. G., Titball, R. W., Peacock, S. J., Cerdeño-Tárraga, A. M., Atkins, T., Crossman, L. C., Parkhill, J. (2004). Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(39), 14240–14245. https://doi.org/10.1073/pnas.0403302101

Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J., & Polz, M. F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science*, *320*(5879), 1081–1085. https://doi.org/10.1126/science.1157890

Hurley, C. C., Quirke, A. M., Reen, F. J., & Boyd, E. F. (2006). Four genomic islands that mark

post-1995 pandemic *Vibrio parahaemolyticus* isolates. *BMC Genomics*, *7*. https://doi.org/10.1186/1471-2164-7-104

Jayaraman, A., & Wood, T. K. (2008). Bacterial Quorum Sensing: Signals, Circuits, and Implications for Biofilms and Disease. *Annual Review of Biomedical Engineering*, *10*(1), 145–167. https://doi.org/10.1146/annurev.bioeng.10.061807.160536

Johnson, C. N. (2013). Fitness Factors in Vibrios: A Mini-review. *Microbial Ecology*, *65*(4), 826–851. https://doi.org/10.1007/s00248-012-0168-x

Johnson, Crystal N., John C. Bowers, Kimberly J. Griffitt, Vanessa Molina, Rachel W. Clostio, Shaofeng Pei, Edward Laws. (2013). Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.01296-12

Jones, J. L., Lüdeke, C. H. M., Bowers, J. C., Garrett, N., Fischer, M., Parsons, M. B., DePaola, A. (2012). Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. *Journal of Clinical Microbiology*, *50*(7), 2343–2352. https://doi.org/10.1128/JCM.00196-12

Jones, S.; Summer-Brason, B. (1998). Incidence and detection of pathogenic Vibrio spp. in a northern New England estuary, USA. *Journal of Shellfish Research*, 17, 1665–1669.

Kaneko and, T., & Colwell2, R. R. (1973). Ecology of Vibrio parahaemolyticus in Chesapeake Bay. *JOURNAL OF BACTERIOLOGY*, *113*(1), 24–32.

Kaneko, T., & Colwell, R. R. (1975). Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. *Appl. Environ. Microbiol*., 30(2), 251-257.

Keymer, D. P., Miller, M. C., Schoolnik, G. K., & Boehm, A. B. (2007). Genomic and phenotypic diversity of coastal *Vibrio cholerae* strains is linked to environmental factors. *Appl. Environ. Microbiol*., 73(11), 3705-3714.

Klein, S. L., Gutierrez West, C. K., Mejia, D. M., & Lovell, C. R. (2014). Genes similar to the *Vibrio parahaemolyticus* virulence-related genes *tdh, tlh,* and *vscC2* Occur in Other vibrionaceae species isolated from a pristine estuary. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.02895-13

Lopez-Hernandez, K. M., Pardio-Sedas, V. T., Lizarraga-Partida, L., Williams, J. de J., Martinez-Herrera, D., Flores-Primo, A., Rendon-Castro, K. (2015). Environmental parameters influence on the dynamics of total and pathogenic *Vibrio parahaemolyticus* densities in *Crassostrea virginica* harvested from Mexico's Gulf coast. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2014.11.015

Lovell, C. R. (2017). Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-017-8096-9

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iida, T. (2003). Genome sequence of *Vibrio parahaemolyticus*: A pathogenic mechanism distinct from that of *V. cholerae*. *Lancet*. https://doi.org/10.1016/S0140-6736(03)12659-1

Martinez-Urtaza, J., Baker-Austin, C., Jones, J. L., Newton, A. E., Gonzalez-Aviles, G. D., & DePaola, A. (2013). Spread of Pacific Northwest *Vibrio parahaemolyticus* Strain. *New England Journal of Medicine*. https://doi.org/10.1056/NEJMc1305535

Martinez-Urtaza, J., Blanco-Abad, V., Rodriguez-Castro, A., Ansede-Bermejo, J., Miranda, A., & Rodriguez-Alvarez, M. X. (2012). Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus* in offshore areas. *The ISME Journal*. https://doi.org/10.1038/ismej.2011.156

Martinez-Urtaza, J., Lozano-Leon, A., Varela-Pet, J., Trinanes, J., Pazos, Y., & Garcia-Martin, O. (2008). Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the rias of Galicia, Spain. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.01307-07

Martinez-Urtaza, J., Van Aerle, R., Abanto, M., Haendiges, J., Myers, R. A., Trinanes, J., Gonzalez-Escalona, N. (2017). Genomic variation and evolution of *Vibrio parahaemolyticus* ST36 over the course of a transcontinental epidemic expansion. *MBio*, *8*(6). https://doi.org/10.1128/mBio.01425-17

Meibom, K. L., Blokesch, M., Dolganov, N. A., Wu, C. Y., & Schoolnik, G. K. (2005). Microbiology: Chitin induces natural competence in vibrio cholerae. *Science*, *310*(5755), 1824–1827. https://doi.org/10.1126/science.1120096

Metzger, L. C., & Blokesch, M. (2014). Composition of the DNA-uptake complex of Vibrio cholerae. *Mobile Genetic Elements*, *4*(1), e28142. https://doi.org/10.4161/mge.28142

Morrow, J. D., & Cooper, V. S. (2012). Evolutionary effects of translocations in bacterial genomes. *Genome Biology and Evolution*. https://doi.org/10.1093/gbe/evs099

Newton et al., 2013. (n.d.).

Nilsson, W. B., Paranjpye, R. N., Hamel, O. S., Hard, C., & Strom, M. S. (2019). Vibrio parahaemolyticus risk assessment in the Pacific Northwest: it's not what's in the water. *FEMS Microbiology Ecology*, *95*(4). https://doi.org/10.1093/femsec/fiz027

Nishibuchi, M., Fasano, A., Russell, R. G., & Kaper, J. B. (1992). *Enterotoxigenicity of Vibrio parahaemolyticus with and without Genes Encoding Thermostable Direct Hemolysin* (Vol. 60).

Oberbeckmann, S., Wichels, A., Wiltshire, K. H., & Gerdts, G. (2011). Occurrence of Vibrio parahaemolyticus and Vibrio alginolyticus in the German Bight over a seasonal cycle. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. https://doi.org/10.1007/s10482-011-9586-x

Oberbeckmann, S., Fuchs, B. M., Meiners, M., Wichels, A., Wiltshire, K. H., & Gerdts, G. (2012). Seasonal dynamics and modeling of a Vibrio community in coastal waters of the North

Sea. Microbial ecology, 63(3), 543-551.

Panicker, G.; Call, D. R.; Krug, M. J.; Bej, A. K. Detection of pathogenic *Vibrio spp*. in shellfish by using multiplex PCR and DNA microarrays. Applied and Environmental Microbiology 2004, 70(12), 7436–44.

Paranjpye, R., Hamel, O. S., Stojanovski, A., & Liermann, M. (2012). Genetic diversity of clinical and environmental *Vibrio parahaemolyticus* strains from the Pacific northwest. *Applied and Environmental Microbiology*, *78*(24), 8631–8638. https://doi.org/10.1128/AEM.01531-12

Paranjpye, R. N., Nilsson, W. B., Liermann, M., Hilborn, E. D., George, B. J., Li, Q., … Sandifer, P. A. (2015). Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA. *FEMS Microbiology Ecology*. https://doi.org/10.1093/femsec/fiv121

Park, K. S., Ono, T., Rokuda, M., Jang, M. H., Okada, K., Iida, T., & Honda, T. (2004). Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infection and Immunity*, *72*(11), 6659–6665. https://doi.org/10.1128/IAI.72.11.6659-6665.2004

Parveen, S., Hettiarachchi, K. A., Bowers, J. C., Jones, J. L., Tamplin, M. L., McKay, R., … DePaola, A. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology*, *128*(2), 354–361. https://doi.org/10.1016/j.ijfoodmicro.2008.09.019

Pruzzo, C., Vezzulli, L., & Colwell, R. R. (2008, June). Global impact of *Vibrio cholerae* interactions with chitin. *Environmental Microbiology*. https://doi.org/10.1111/j.1462-2920.2007.01559.x

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(5), 1528–1533. https://doi.org/10.1073/pnas.0510322103

Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio parahaemolyticus* in Raw Oysters | FDA. (n.d.). Retrieved October 29, 2019, from https://www.fda.gov/food/cfsan-risk-safety-assessments/quantitative-risk-assessment-public-health-impact-pathogenic-vibrio-parahaemolyticus-raw-oysters

Raghunath, P. (2014). Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2014.00805

Semenza, J. C., Trinanes, J., Lohr, W., Sudre, B., Löfdahl, M., Martinez-Urtaza, J., Rocklöv, J. (2017). Environmental suitability of vibrio infections in a warming climate: An early warning system. *Environmental Health Perspectives*, *125*(10). https://doi.org/10.1289/EHP2198

Shapiro, B. J., & Polz, M. F. (2014). Ordering microbial diversity into ecologically and genetically cohesive units. *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2014.02.006

Shinoda, S., & Miyoshi, S. ichi. (2006). Hemolysins of vibrio cholerae and other vibrio species. In *The Comprehensive Sourcebook of Bacterial Protein Toxins* (pp. 748–762). Elsevier Inc. https://doi.org/10.1016/B978-012088445-2/50049-4

Sun, F., Zhang, Y., Wang, L., Yan, X., Tan, Y., Guo, Z., Zhou, D. (2012). Molecular Characterization of Direct Target Genes and cis-Acting Consensus Recognized by Quorum-Sensing Regulator AphA in *Vibrio parahaemolyticus*. *PLoS ONE*, *7*(9). https://doi.org/10.1371/journal.pone.0044210

Takemura, A. F., Chien, D. M., & Polz, M. F. (2014). Associations and dynamics of vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2014.00038

Tamames, J., Sánchez, P. D., Nikel, P. I., & Pedrós-Alió, C. (2016). Quantifying the relative importance of phylogeny and environmental preferences as drivers of gene content in prokaryotic microorganisms. *Frontiers in Microbiology*, *7*(MAR). https://doi.org/10.3389/fmicb.2016.00433

Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., Lim, E., & Polz, M. F. (2004). Diversity and dynamics of a North Atlantic coastal Vibrio community. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.70.7.4103-4110.2004

Tiruvayipati, S., & Bhassu, S. (2016, April 25). Host, pathogen and the environment: The case of *Macrobrachium rosenbergii, Vibrio parahaemolyticus* and magnesium. *Gut Pathogens*. BioMed Central Ltd. https://doi.org/10.1186/s13099-016-0097-1

Tiruvayipati, S., Bhassu, S., Kumar, N., Baddam, R., Shaik, S., Gurindapalli, A., Ahmed, N. (2013). Genome anatomy of the gastrointestinal pathogen, *Vibrio parahaemolyticus* of crustacean origin. *Gut Pathogens*. https://doi.org/10.1186/1757-4749-5-37

Tonkin, J. D., Bogan, M. T., Bonada, N., Rios-Touma, B., & Lytle, D. A. (2017). Seasonality and predictability shape temporal species diversity. *Ecology*, *98*(5), 1201–1216. https://doi.org/10.1002/ecy.1761

Turner, J. W., Berthiaume, C. T., Morales, R., Armbrust, E. V., & Strom, M. S. (2016). Genomic evidence of adaptive evolution in emergent *Vibrio parahaemolyticus* ecotypes. *Elementa: Science of the Anthropocene*. https://doi.org/10.12952/journal.elementa.000117

Turner, J. W., Malayil, L., Guadagnoli, D., Cole, D., & Lipp, E. K. (2014). Detection of *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae* with respect to seasonal fluctuations in temperature and plankton abundance. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12246

Urmersbach, S., Alter, T., Koralage, M. S. G., Sperling, L., Gerdts, G., Messelhäusser, U., & Huehn, S. (2014). Population analysis of *Vibrio parahaemolyticus* originating from different geographical regions demonstrates a high genetic diversity. *BMC Microbiology*, *14*, 59. https://doi.org/10.1186/1471-2180-14-59

Urquhart, E. A., Jones, S. H., Yu, J. W., Schuster, B. M., Marcinkiewicz, A. L., Whistler, C. A., & Cooper, V. S. (2016). Environmental Conditions Associated with Elevated Vibrio parahaemolyticus Concentrations in Great Bay Estuary, New Hampshire. https://doi.org/10.1371/journal.pone.0155018

Venkateswaran, K., Kiiyukia, C., Nakanishi, K., Nakano, H., Matsuda, O., & Hashimoto, H. (1990). The role of sinking particles in the overwintering process of *Vibrio parahaemolyticus* in a marine environment. FEMS Microbiology Ecology, 6(2), 159-166.

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P. C., Colwell, R. R., Höfle, M. G., & Pruzzo, C. (2012). Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. *The ISME Journal*. https://doi.org/10.1038/ismej.2011.89

Vezzulli, L., Pezzati, E., Moreno, M., Fabiano, M., Pane, L., Pruzzo, C., & The Vibrio Sea Consortium. (2009). Benthic ecology of Vibrio spp. and pathogenic Vibrio species in a coastal Mediterranean environment (La Spezia Gulf, Italy). *Microbial Ecology*. https://doi.org/10.1007/s00248-009-9542-8

Weinert, L. A., & Welch, J. J. (2017, December 1). Why Might Bacterial Pathogens Have Small Genomes? *Trends in Ecology and Evolution*. Elsevier Ltd. https://doi.org/10.1016/j.tree.2017.09.006

Whistler, C. A., Hall, J. A., Xu, F., Ilyas, S., Siwakoti, P., Cooper, V. S., & Jones, S. H. (2015). Use of whole-genome phylogeny and comparisons for development of a multiplex PCR assay to identify sequence type 36 Vibrio parahaemolyticus. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.00034-15

Williams, T. C., Ayrapetyan, M., & Oliver, J. D. (2014). Implications of chitin attachment for the environmental persistence and clinical nature of the human pathogen *Vibrio vulnificus. Appl. Environ. Microbiol.*, 80(5), 1580-1587.

Wong, H. C., & Wang, P. (2004). Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of applied microbiology*, 96(2), 359-366.

Wong, H. C., Peng, P. Y., Han, J. M., Chang, C. Y., & Lan, S. L. (1998). Effect of mild acid treatment on the survival, enteropathogenicity, and protein production in *Vibrio parahaemolyticus*. *Infection and immunity*, 66(7), 3066-3071.

Xu, F., Gonzalez-Escalona, N., Haendiges, J., Myers, R. A., Ferguson, J., Stiles, T., Whistler, C. A. (2017). Sequence type 631 *Vibrio parahaemolyticus*, an emerging foodborne pathogen in North America. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.02162-16

Xu, F., Ilyas, S., Hall, J. A., Jones, S. H., Cooper, V. S., & Whistler, C. A. (2015). Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2015.00272

Young, I., Gropp, K., Fazil, A., & Smith, B. A. (2015). Knowledge synthesis to support risk assessment of climate change impacts on food and water safety: A case study of the effects of water temperature and salinity on *Vibrio parahaemolyticus* in raw oysters and harvest waters. *Food Research International*, 68, 86-93.

Zhang, L., & Orth, K. (2013). Virulence determinants for *Vibrio parahaemolyticus* infection. *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2013.02.002

Zhang, Y., Gao, H., Osei-Adjei, G., Zhang, Y., Yang, W., Yang, H., … Zhou, D. (2017). Transcriptional regulation of the type VI secretion system 1 genes by quorum sensing and ToxR in Vibrio parahaemolyticus. *Frontiers in Microbiology*, *8*(OCT). https://doi.org/10.3389/fmicb.2017.02005

Zhang, Y., Qiu, Y., Tan, Y., Guo, Z., Yang, R., & Zhou, D. (2012). Transcriptional regulation of *opaR, qrr2-4* and *aphA* by the master quorum-sensing regulator *opaR* in Vibrio parahaemolyticus. *PLoS ONE*, *7*(4). https://doi.org/10.1371/journal.pone.0034622

Zimmerman, A. M., DePaola, A., Bowers, J. C., Krantz, J. A., Nordstrom, J. L., Johnson, C. N., & Grimes, D. J. (2007). Variability of total and pathogenic *Vibrio parahaemolyticus* densities in Northern Gulf of Mexico water and oysters. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.01700-07

Chapter 1

Article

Forecasting Seasonal *Vibrio parahaemolyticus* Concentrations in New England Shellfish

Meghan A. Hartwick[1,2], Erin A. Urquhart[1,3] , Cheryl A. Whistler[1,2], Vaughn S. Cooper[4], Elena N. Naumova[5], Stephen H. Jones[1,2,3,*]

1 Northeast Center for Vibrio Disease and Ecology, University of New Hampshire, Durham, NH; mah2002@wildcats.unh.edu; Urquhart.erin@epa.gov
2 Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH; Cheryl.Whistler@unh.edu
3 Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH; Stephen.Jones@unh.edu
4 Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA; vaughn.cooper@pitt.edu
5 Division of Nutrition Data Sciences, Friedman School of Nutrition Science and Policy, Tufts University, Boston, MA; elena.naumova@tufts.edu

* Correspondence: stephen.jones@unh.edu (S.H.J.)

Received: date; Accepted: date; Published: date

Abstract: Seafood-borne *Vibrio parahaemolyticus* illness is a global public health issue facing resource managers and the seafood industry. The recent increase in shellfish-borne illnesses in the Northeast United States has resulted in application of intensive management practices based on a limited understanding of when and where risks are present. We aim to determine the contribution of factors that affect *V. parahaemolyticus* concentrations in oysters (*Crassostrea virginica*) using ten-years of surveillance data for environmental and climate conditions in the Great Bay Estuary of New Hampshire from 2007 to 2016. Time series analysis was applied to analyze *V. parahaemolyticus* concentrations and local environmental predictors and develop predictive models. Whereas many environmental variables correlated with *V. parahaemolyticus* concentrations, only a few retained significance in capturing trends, seasonality and data variability. The optimal predictive model contained water temperature and pH, photoperiod, and the calendar day of study. The model enabled relatively accurate seasonality-based prediction of *V. parahaemolyticus* concentrations for 2014-2016 based on the 2007-2013 dataset and captured the increasing trend in extreme values of *V. parahaemolyticus* concentrations. The developed method enables informative tracking of *V. parahaemolyticus* concentrations in coastal ecosystems and presents a useful platform for developing area-specific risk forecasting models.

Keywords: *Vibrio parahaemolyticus*; seasonality; seafood illness; forecasting; climate change

1. Introduction

Vibrio parahaemolyticus is the leading cause of seafood-borne gastroenteritis in the US and worldwide [1-3]. Most strains are believed to be non-pathogenic and the strains that do cause gastroenteritis and septicemia in humans have been historically associated with warm water environments [4-6]. Over the past decade, however, illnesses caused by *V. parahaemolyticus* have become more frequent in some cold and temperate water environments where illnesses were previously rare [7-14]. This new pattern of *V.*

parahaemolyticus disease likely stems from a combination of observed trends, such as introduced and ecosystem establishment of pathogenic strains, increased summertime production and consumption of raw shellfish, and climate related changes causing warmer sea surface temperatures and more variable salinities [7-8,13-19]. In the Northeast United States (US) where pathogenic *V. parahaemolyticus* are now established, foodborne illness is most frequently acquired from the consumption of raw or undercooked shellfish [3]. Post-harvest management has effectively reduced the incidence of *V. parahaemolyticus* disease outbreaks in this region. However, illness still occurs and achieving effective post-harvest control is both resource and time intensive. Effective pre-harvest *V. parahaemolyticus* forecasting tools would be valuable to shellfish growers and managers alike to make informed decisions about the *V. parahaemolyticus* risk conditions at the time of harvest and potentially reduce the risk and cost of *V. parahaemolyticus* management.

V. parahaemolyticus is a naturally occurring bacterial species that persists in a wide range of conditions in most marine and estuarine environments [5,20-30]. In multiple studies, temperature and salinity correlate most strongly with *V. parahaemolyticus*, but the strength of this relationship varies by region and season [31]. Similarly, nutrients, chlorophyll *a*, pH and turbidity, were inconsistent and depended on the region and the variability of these factors. Therefore, region and even harvest area specific studies are necessary to provide an accurate description of the influence of environmental conditions on *V. parahaemolyticus* concentration [32].

Long-term monitoring has been established in the Great Bay Estuary (GBE) by the Northeast Center for *Vibrio* Disease and Ecology at the University of New Hampshire (UNH) since 2007 [33-36]. The GBE is located on the border of New Hampshire and Maine (Figure 1) and has a long history of studies on pathogenic *Vibrio* spp. [37-39]. It is a regionally significant estuary that experiences wide ranging environmental, climatic, and biological conditions [10], and thus serves as a useful model representative of regional estuaries. It is unique in that *V. parahaemolyticus* illnesses are still rare [40], although the *V. parahaemolyticus* population in the Northeast is evolving [13,14] and commercial shellfish harvests are rapidly increasing. The ongoing surveillance enables the development of pre-harvest risk-forecasting models.

Figure. 1. Study area and sites for oyster and water sampling in the Great Bay Estuary, New Hampshire, USA. OR = Oyster River; NI = Nannie Island.

The goal of this study was to develop an integrated modeling approach to predict *V. parahaemolyticus* concentrations in shellfish at a pre-harvesting stage as a tool for managing this significant public health issue. We used data from 2007-2016 to capture long-term trends, seasonal fluctuations in a broad range of environmental and climatic predictors of *V. parahaemolyticus* dynamics aiming to create a model development approach that could be transferable to other estuaries.

2. Materials and Methods

2.1. Study sites, environmental sampling and bacterial analysis

The study area was the Great Bay estuary in New Hampshire. The two sampling locations (Figure 1) were near Nannie Island (NI) in Great Bay where shellfish harvest classification is Approved, and a site in the tidal portion of the Oyster River (OR), where harvesting is Prohibited because of proximity to the Durham NH wastewater treatment facility. Both are locations of significant oyster (*Crassostrea virginica*) beds and long-term monitoring locations [36, 38] and have different ecosystem and environmental conditions. The estuary has been monitored for over 30 consecutive years (March-December) through efforts by multiple agencies, including the Great Bay National Estuarine Research Reserve (GBNERR) and their System Wide Monitoring Program (SWMP; https://cdmo.baruch.sc.edu/dges/). The average temperature, salinity, dissolved oxygen (DO), pH, and turbidity data were calculated from continuous (Q15) measurements obtained from the SWMP database for 2007-2016 for times simultaneous with and preceding oyster sampling in 12-hour periods to account for ecological lag times and capture a more complete assessment of the potential environmental conditions that may have contributed *V. parahaemolyticus* concentrations observed at the time of collection. Monthly SWMP samples provided nutrient (total dissolved nitrogen (TDN) and chlorophyll *a* (concentration by fluorescence; CHL) data for monitoring sites in close proximity to the NI and OR sampling locations. Meteorological data were acquired from several weather stations (SWMP; UNH) in the Great Bay region. Water quality parameters were measured *in situ* at the time of sampling using calibrated YSI 6600 and EXO multiprobe datasondes (Yellow Springs Instruments, Yellow Springs, Ohio).

2.2. Oyster sample collection and processing

Oyster samples were collected from the two oyster beds at NI and OR except during January-March from June 2007 through December 2016. For each sampling date, 10-12 oysters were cleaned and aseptically shucked into a sterile beaker (liquor and meat), weighed and diluted 1:1 with alkaline peptone water (APW (pH 8.6, 1% NaCl), and homogenized. 20mL homogenate was further diluted in 80mL APW for a starting dilution of 1:10. 1 mL of 1:10 solution was added to three tubes and then serially diluted with 1mL aliquots into a three serial dilution containing 9 ml of APW (pH 8.6, 1% NaCl). Each tube was incubated at 37°C overnight (18-20h) following the FDA Bacteriological Analytical Manual (BAM)[41].

Following incubation, turbid APW tubes were scored positive for growth. From 2007-2010, turbid tubes were streaked to TCBS (Thioglycollate-Citrate-Bile-Salts) agar (Beckton Dickson (BD), Franklin Lakes, NJ) and incubated at 37°C for 18 –20 h. From 2011-2016, turbid tubes were streaked onto Vibrio CHROMAgar (CHROMagar, Paris, France) and incubated at 37°C for 18 –20 h. Sucrose negative (green) colonies from TCBS or purple colonies from CHROMagar were streaked onto tryptic soy agar (TSA; BD) and incubated at room temperature for 18 – 20 h. TSA isolates were inoculated in Heart Infusion (HI) broth for 18-20 h. 1mL HI aliquots were then pelleted for 5 min at 8000rpm, re-suspended in 1 mL molecular biology grade water (Phenix Research Products), boiled at 100 °C for 10 min and debris removed by centrifugation. Species identity of isolates was determined by polymerase chain reaction (PCR) performed using 2μL of the cleared supernatant in 13 μL Mastermix, iQSupermix (BioRad, Hercules, CA) using a BIO

RAD T100 thermocycler and published primers and conditions [42] for 2007-2014; [19] with slight modifications for 2015-2016. The PCR amplicons were visualized on 1.2% agarose gel with addition of Gel Red (Phenix Research Products, Candler, NC) under UV light. Concentration (Most Probable Number) was calculated from *V. parahaemolyticus* species-specific gene (tlh) confirmed isolates from enrichment tubes and the BAM MPN tables.

2.3. Statistical analysis

All statistical computations were performed in the R Statistical Program and Environment, version 3.5.1 [43] with add-on packages MGCV [52]. Graphics were produced with ggplot2 [53]. Multiple steps of data analysis were performed to evaluate the relationship between environmental determinants, seasonality and *V. parahaemolyticus* concentrations in the GBE. MPN values for *V. parahaemolyticus* concentrations were log-transformed for analysis and model development to approximate normality and reduce skewness. Sampling events with missing environmental measurements (n=29), when *V. parahaemolyticus* was not detected (n=71) and one sample that exceeded >2 standard deviations were excluded from concentration model development. Statistical significance for all analysis in this study was determined using an alpha level of $p < 0.05$.

2.3.1. Model development strategy

All measurements were arranged in chronological order based on the date of measurement and multiple time series were compiled for the entire study period. The relationship between the time series for water quality variables, including water temperature, salinity, pH, DO, turbidity, CHL, TDN, rainfall and *V. parahaemolyticus* concentrations in oysters were evaluated using correlation and regression analysis. We used log-transformed values of *V. parahaemolyticus* concentrations (Y_t) and applied a Gaussian family distribution with an identity link function relating the expected value of response variable Y_t to selected predictors [44,45]. Transformation of water temperature, salinity, pH, DO, turbidity, CHL, TDN and rainfall was also explored as response variables in seasonality analysis and as predictor variables for *V. parahaemolyticus* in regression analysis with log or log + 1. We assessed the shape of relationships (linear and non-linear) between *V. parahaemolyticus* concentrations in oysters and environmental predictors. Variables that were significant in univariate regression were used to develop multiple regression models. We also assessed seasonality and trends over time and explored alternative variables representing seasonality with respect to their ability to improve the stability of forecasting. Assumptions of inter-correlation among predictors were evaluated using Spearman correlation analysis. Below we provide the detailed description of model building.

2.3.2. Seasonality and trend analysis

To explore the seasonality and the general trend throughout the whole study period (2007-2016) in all variables: *V. parahaemolyticus* concentrations, temperature, DO, salinity, pH, turbidity, CHL, TDN and rainfall, we develop two models with different ways of presenting the periodicity of seasonal oscillations. Model 1 contains variables for a linear trend and photoperiod. Model 2 uses terms for a linear trend and harmonic regression terms for the calendar day in the study as follows:

$$\text{Model 1: } E(Y_t) = \beta_0 + \beta_1 t + \beta_p Photoperiod, \quad (1)$$

$$\text{Model 2: } E(Y_t) = \beta_0 + \beta_1 t + \beta_s \sin(2\pi\omega t) + \beta_c \cos(2\pi\omega t). \quad (2)$$

In both models, Y_t is the daily time series for the outcome of interest, β_0 is the intercept, t is the daily time series, β_1 indicated a general trend in the outcome of interest; βs and βc are the coefficients of the harmonic terms and ω is the term representing the annual cycle (365.25 days, ω=1/365.25). The harmonic terms in

Model 2 are expected to depict the periodic oscillation that can be also captured by the β_P in Model 1. The phase shift of periodic oscillations identified by Model 2 was determined as follows:

$$\psi = \arctan\left(\frac{\hat{\beta}_S}{\hat{\beta}_C}\right) + k. \quad (3)$$

When estimates of $\hat{\beta}_S$ and $\hat{\beta}_c$ were positive, k = 0. If $\hat{\beta}_S < 0$ and $\hat{\beta}_c > 0$, then $k = 2\pi$. If $\hat{\beta}_S$ and $\hat{\beta}_c$ were negative, or if $\hat{\beta}_S > 0$ and $\hat{\beta}_c < 0$, then $k = \pi$. The phase shift (ψ) was multiplied by 365.25 days in order to calculate peak timing. Covariance of β_s and β_c ($\sigma_{\beta_c\beta_s}$) and variance of β_s and β_c ($\hat{\beta}_s^2$ and $\hat{\beta}_c^2$) estimated the variance of the phase shift (ψ) as:

$$var(\psi) = \frac{(\sigma_{\beta s}\beta c)^2 + (\sigma_{\beta c}\beta s)^2 - (2\sigma_{\beta_c\beta_s}\beta_s\beta_c)}{(\hat{\beta}_s^2 + \hat{\beta}_c^2)(\hat{\beta}_s^2 + \hat{\beta}_c^2)^2} \quad , \quad (4)$$

and confidence intervals of the peak timing were determined as: $1.96 * \sqrt{Var(\psi) * 365.25/2\pi}$. Secular trends were assessed using nine default thin-plate splines (f) from the MGCV package in R [46] in Model 3 and Model 4 as shown

$$\text{Model 3: } E(Y_t) = \beta_0 + \beta_1 f(t) + \beta_p Photoperiod, \quad (5)$$

$$\text{Model 4: } E(Y_t) = \beta_0 + \beta_1 f(t) + \beta_s \sin(2\pi\omega t) + \beta_c \cos(2\pi\omega t). \quad (6)$$

The models' performance was determined by the deviance explained, residual variation, AIC, and coefficient of determination (r^2) value. The trend term was determined to be non-linear based on visual assessment, positive Δ AIC and positive Δ r^2 and Δ Deviance > 0.1.

2.3.2. Extreme value trend analysis

In addition to a general trend and Mann-Kendall trend analysis, we explored potential trends in high values of *V. parahaemolyticus* concentration as well as TDN, pH and salinity based on their importance in multiple regression models to estimate *V. parahaemolyticus* concentration by determining the number of events when the observations were above its 75th percentile. For other variables, trends were evaluated using the number of observations within the 25th and 75th percentile.

2.3.3. Variable selection and non-linearity assessment

To explore the relationship between the response variable, *V. parahaemolyticus* concentrations, and predictor variables, we incorporated each environmental parameter individually into linear (Model 5) and non-linear (Model 6) regression models. These two models were applied to the log-transformed values of *V. parahaemolyticus* concentrations (Y_t):

$$\text{Model 5: } E(Y_t) = \beta_0 + \beta_1 X_t, \quad (7)$$

where Y_t is the daily time series for the log-transformed *V. parahaemolyticus* concentrations in oyster, , X_t is the daily time series for an environmental predictor and β_1 reflects the degree of captured linear relation in the daily time series of response and predictor variables.

Non-linear relationships were initially assessed using nine default thin-plate splines (f) from the MGCV package in R [46] as shown in

$$\text{Model 6: } E(Y_t) = \beta_0 + \beta_1 f(X_t). \quad (8)$$

The relationships between the environmental conditions and *V. parahaemolyticus* concentrations were overlaid with loess curves to visualize the relationship. Non-linear relationships were evaluated by the differences between the significance of the coefficient, residual variation, AIC, and coefficient of determination (r^2) value. Positive values indicate that the measure improved in Model 6 compared to Model

5 and negative values indicate a decrease in the model evaluation measurement. Variables were determined to be non-linear based on visual assessment, positive Δ AIC and positive Δ r^2 and Δ Deviance > 0.1. When strong non-linear non-monotonic relationships were detected, we re-parametrized the predictor by centering the variable around its *V. parahaemolyticus* concentration maximum and created a new variable to provide biological interpretability to the model [47]. For example, a new variable for pH was created by squaring the difference between the observed pH values and the value of 7.8 selected for the centering. Re-parametrized variables are indicated as C-variable name (e.g., C-pH).

2.3.4. Model building

The environmental parameters determined to be significant in univariate models (Models 5 and 6) were incorporated into a multivariate general linear regression model using Gaussian (GLM-G) and negative binomial (GLM-NB) distributional assumptions. For GLM-NB, the dispersion was determined by the index of dispersion: $\emptyset = variance/mean = 1$, where $\emptyset < 1$ refers to under-dispersion and $\emptyset > 1$ refers to over-dispersion. We started with the sequential model building (Model 7):

$$\text{Model 7: } E(Y_t) = \beta_0 + \beta_1 X_{1,t} + \beta_k X_{k,t}\ , \tag{9}$$

where Y_t is the daily time series for the outcome of interest, β_0 is the intercept and t is the daily time series; $X_{1,t}$ ... $X_{k,t}$ are the daily time series for environmental predictors, including the reparametrized centered variables and interaction terms; β_1 ... β_k are the corresponding coefficients.

We then added variables to reflect the trend and seasonal oscillations and fine-tuned the model by using the photoperiod variable (Model 8), or harmonic terms (Model 9). In both models:

$$\text{Model 8: } E(Y_t) = \beta_0 + \beta_1 X_{1,t} + \beta_2 X_{2,t} + \cdots \beta_l t + \beta_p Photoperiod, \tag{10}$$

$$\text{Model 9: } E(Y_t) = \beta_0 + \beta_1 X_{1,t} + \beta_2 X_{2,t} + \cdots \beta_l t + \beta_s \sin(2\pi\omega t) + \beta_c \cos(2\pi\omega t), \tag{11}$$

where Y_t is the daily time series for the outcome of interest, β_0 is the intercept and t is the daily time series; $X_{1,t}$ ... $X_{k,t}$ are the daily time series for the selected environmental predictors, including the reparametrized centered variables and interaction terms; β_1 ... β_l are the corresponding coefficients. In Model 8 and 9, β_p is the coefficient of the photoperiod variable. In Model 10, βs and βc are the coefficients of the harmonic terms and ω is the term representing the annual cycle (365.25 days), as in Model 2.

For these hybrid models, we employed sequential model building using both Gaussian and negative binomial distributional assumptions in parallel and explored the contribution of interaction terms to the model's fit. Overall performance of GLMs was evaluated by evaluation of Akaike's Information Criterion (AIC) [48], residual variation, and deviance explained to determine the number and combination of variables that provided the strongest fit for the full time period of 2007-2016. Model fit was evaluated by the differences between the significance of the coefficient, residual variation, AIC, and coefficient of determination (r^2) value. Model selection was based on AIC value and improvement of r^2 and deviance explained > 0.1.

Using the parameters of the harmonic terms, e.g. the estimates of β_s and β_c regression coefficients and their error from the Model 9, we applied the δ-method [49,50] to estimate seasonal peak timing along with its error term, expressed in days.

2.4. Assessment of model forecasting ability

The predictive skill or forecasting ability of the selected versions of Models 7, 8 and 9 models were evaluated by splitting the whole dataset into two datasets representing two periods: a training dataset from 2007-2013, and a test dataset from 2014-2016. Correlations between environmental variables and *V. parahaemolyticus* concentrations were compared for the full, training, and testing intervals. The forecasting

ability and model performance were determined by coefficient of determination (r^2), and overall residual deviance. Forecasting error was evaluated by root mean square error (RMSE)

3. Results

3.1 V. parahaemolyticus concentrations in the GBE, 2007-2016

V. parahaemolyticus was detected in 144 oyster samples during May through December from June 27, 2007 to December 5, 2016 for both the NI (n=77) and OR (n=67) study sites that included complete sets of data for environmental variables. There were no significant between-site differences for *V. parahaemolyticus* concentrations or measured condition parameters (data not shown) at the two sites, so all the following analyses use a combined-site database. Our analysis of the samples from 2007-2016 included detection of *tdh* and *trh*, the traditional indicator markers for presence of 'pathogenic' *V. parahaemolyticus*, however, these markers were only detected in two samples during 2009 and were not detected again until 2015. Thus, our study focused on total *V. parahaemolyticus* concentrations because it is important to understand the ecosystem dynamics of the population of this species in shellfish harvest areas [36] as a proxy for risk assessment, and, in part because not all *V. parahaemolyticus* strains in clinical cases in the Northeast US and elsewhere contain either *tdh* or *trh*. Three main aspects of the full ten-year database are the marked seasonality, upward trend in high concentration values, and the wide variability/dispersion of *V. parahaemolyticus* concentrations between years and within each year (Figure 2). The observed *V. parahaemolyticus* concentrations were highly seasonal, ranging from 0.036 MPN/g oyster tissue during cold seasonal conditions to 4600 MPN/g during warm summertime conditions. The highest annual *V. parahaemolyticus* concentrations were higher during the later years than in early years. The detailed analysis of the trends and seasonality is presented below.

3.1.1 Trends and seasonality

V. parahaemolyticus concentrations in oysters and environmental variables in the GBE were formally assessed for seasonality by using a photoperiod (Model 1) and a harmonic regression model (Model 2). These models allowed us to determine whether the study variables displayed re-occurring periodicity and a linear trend using calendar day of study to assess change over time. We examined trends, peak timing and seasonal oscillations in water temperature, DO, salinity, pH, turbidity, CHL, TDN and rainfall. The patterns of data had various shapes, including an extended period of *V. parahaemolyticus* detection during fall compared to spring. The variability in *V. parahaemolyticus* concentration in oysters, water temperature, DO and salinity were highly seasonal and well detected by both photoperiod and harmonic regression models, though the harmonic regression model provided a better fit in all instances (Table 1).

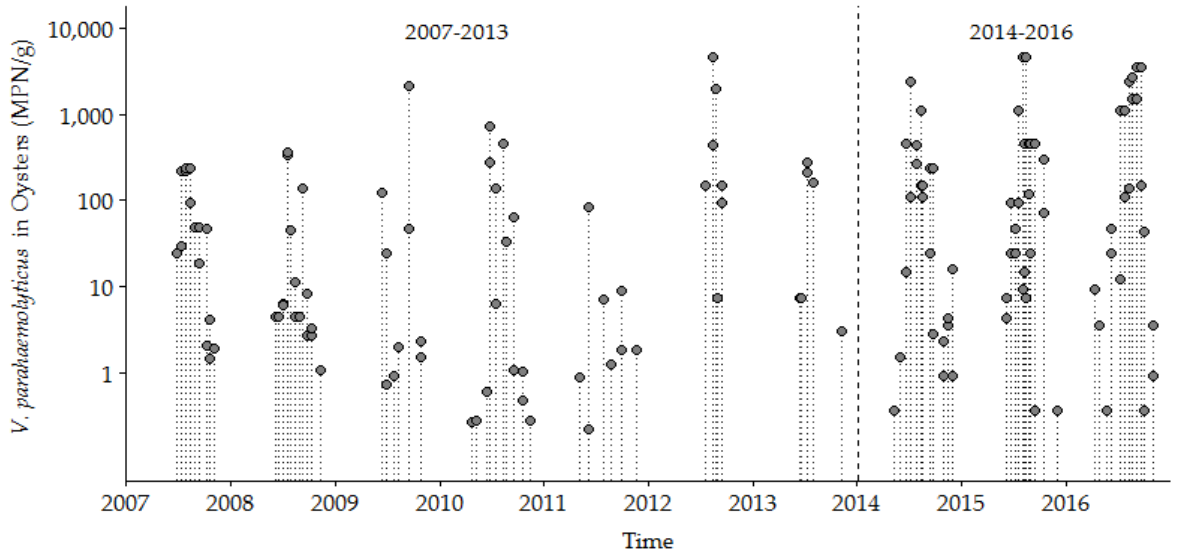


Figure 2. *V. parahaemolyticus* concentrations in oysters from NI and OR at low tide in the GBE in 2007-2016.

Table 1. Trend and seasonality estimates detected by Model 1 and Model 2 for *V. parahaemolyticus* concentrations and environmental variables (Model 1, top and Model 2, bottom).

Variable [a]	Coefficients [b]		Standard Error		r^2	Deviance	AIC	Peak Timing [c]
	Trend	Seasonality	Trend	Seasonality				
Vp (MPN/g)	0.0005***	0.57***	0.0001	0.11	0.19	0.21	673.4	
	0.0006***	-2.87*** -3.66***	0.0001	0.34 0.33	0.50	0.51	597.4	222 ± 5
Water Temperature (°C)	< 0.001	2.01***	< 0.001	0.15	0.53	0.54	774.1	
	0.002*	-5.81*** -10.22***	< 0.001	0.24 0.23	0.93	0.93	497.9	213 ± 2
Dissolved Oxygen (mg/L)	< 0.001	-0.31***	< 0.001	0.05	0.22	0.23	441.5	
	< 0.001	1.45*** 1.91***	< 0.001	0.15 0.14	0.58	0.59	352.0	220 ± 6
Salinity (ppt)	0.001***	-0.19	0.0003	0.20	0.12	0.13	849.4	
	0.002***	-4.06*** -1.77**	0.0003	0.76 0.72	0.26	0.28	825.5	251 ± 18
pH	< 0.001***	-0.02*	< 0.001	0.01	0.08	0.10	19.9	
	< 0.001***	-0.06 0.03	0.006	0.05 0.05	0.09	0.11	20.9	298 ± 98
Turbidity (NTU)	-0.02***	3.93	0.007	4.10	0.06	0.09	1723.6	
	-0.02***	-6.34 -9.83	0.007	16.77 15.87	0.06	0.08	1716.5	135 ± 111
Chlorophyll-*a* (µg/L)	-0.0002	0.62***	0.005	0.0002	0.09	0.10	775.3	
	< 0.001	0.11 -2.02***	<0.001	0.65 0.61	0.09	0.10	778.2	180 ± 37
Total Dissolved Nitrogen (mg/L)	< 0.001***	-0.008*	<0.001	0.005	0.15	0.16	-229.0	
	< 0.001***	0.02 0.04*	< 0.001	0.02 0.02	0.15	0.17	-228.2	206 ± 45

Rainfall (mm)	< 0.001	0.01*	< 0.001	<0.001	0.01	0.02	-76.7	
	< 0.001	-0.03 -0.07**	< 0.001	0.001 < 0.001	0.01	0.04	-74.6	209 ± 38

a Variable are shown for Model 1, top row and Model 2, two bottom rows for sine and cosine terms; b significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1; c Peak timing estimates are represented by the mean and standard error values; for two parameters DO and TDN the estimates reflect the seasonal nadir.

The peak timing of *V. parahaemolyticus* (day 222 ± 5) was determined to be approximately 10 days after the peak timing of water temperature that occurred at day 213 ± 2. The peak timing of salinity and pH were within 25 days of the peak timing of *V. parahaemolyticus*, though as the strength of the seasonality of the variable decreased the confidence intervals around the corresponding the peak timing was observed to increase. Neither model offered a fit to the variability observed in other variables For instance, less than 4% of the variability in rainfall was attributed to seasonality. Rainfall and turbidity measurements above zero were episodic and model fit did not improve above 1% variance explained with log or log + 1 transformation. Figure 3 provides an explanation for the model fit by superimposing daily values for each year and depicting seasonal patterns for each variable.

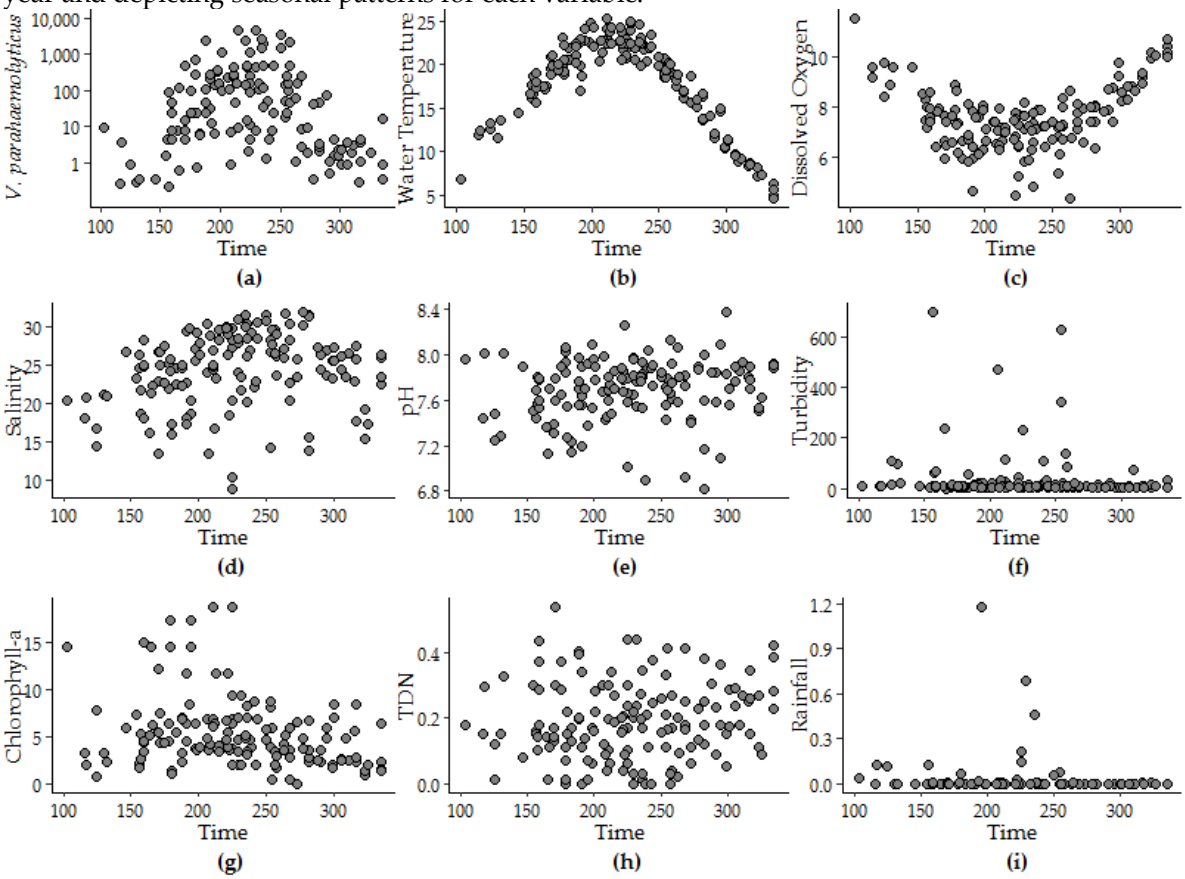


Figure 3. Patterns in **(a)** *V. parahaemolyticus* concentration, **(b)** water temperature, **(c)** dissolved oxygen, **(d)** salinity, **(e)** pH, **(f)** turbidity, **(g)** CHL, (h) TDN, and **(i)** rainfall versus day of the calendar day of the year superimposed from 2007-2016.

Over the ten-year period of surveillance there were significant increases in *V. parahaemolyticus* concentrations, salinity, pH and TDN. Only turbidity decreased during this same period (Table 1). To further explore these findings, we used thin-plate splines to assess secular trends (Table S1) and examined

the trend in extreme values for salinity, pH, TDN, and *V. parahaemolyticus* concentrations that were above the 75% percentile (Table 2; Figure 4). The improvement in fit from the non-linear trend term in the photoperiod model (Model 1 and Model 3) was not seen in the harmonic regression model (Model 2 and Model 4). The change in fit can be largely attributed to interannual variation that was accounted for in the harmonic regression model and so a linear trend term was applied moving forward. For pH, the range of observed pH values decreased over time with more observations occurring within the pH range of 7.56 (25th percentile) to 7.88 (75th percentile). For salinity and pH, more than 55% and 62.1% of days observed in 2015 were above 27.0 ppt for salinity and within the 7.56-7.88 pH range, respectively. TDN above 0.27mg/L was observed in in six of ten years of the study. In later years, at least 45.0% percent of measured TDN was above the 75% percentile. Yearly *V. parahaemolyticus* concentrations above 240 MPN/g oyster tissue increased from 11.8% of samples in 2007 to 38.1% in 2016. Kendall-Mann trend analysis identified significant upward trends in extreme values for *V. parahaemolyticus*, salinity, pH, and TDN ($p < 0.05$).

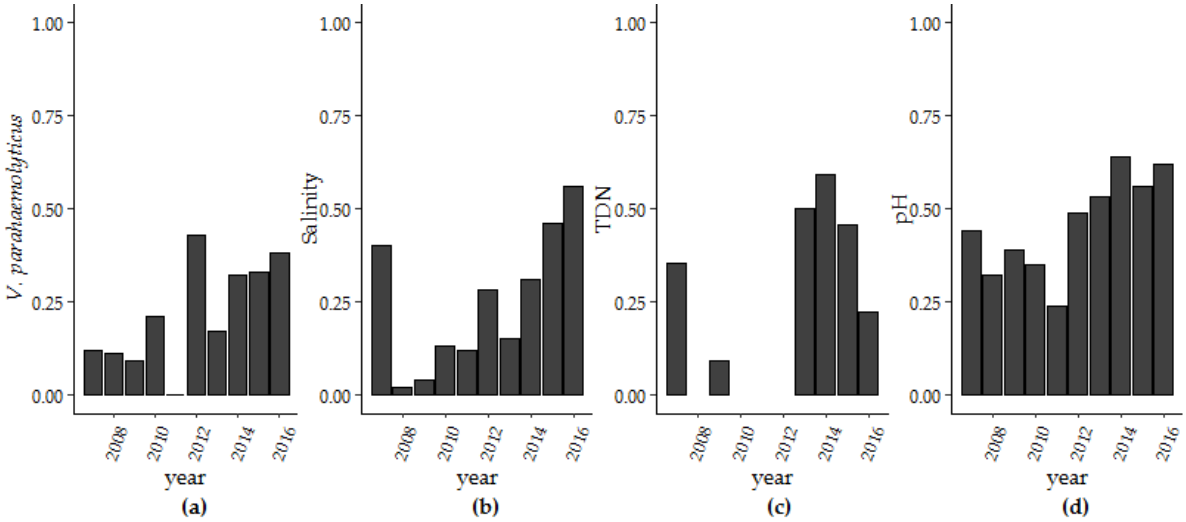


Figure 4. The number of observations per year above the 75th percentile for **(a)** *V. parahaemolyticus* concentrations, **(b)** salinity, **(c)** TDN and between the 25th and 75th percentile for **(d)** pH.

Table 2. Trends of the frequency of days when *V. parahaemolyticus* concentrations, water temperature and salinity exceeded the 75th percentile of data and pH data fall within the 25th to 75th percentile range in GBE during 2007 – 2016.

Year	*V. parahaemolyticus*		Salinity		TDN		pH	
	75th Percentile						25th and 75th percentile	
	220 MPN/g		27 ppt		0.27 mg/L		7.56 - 7.88	
	n	%	n	%	n	%	n	%
2007	2/17	11.8%	196/488	40.2%	6/17	35.3%	215/488	44.1%
2008	2/18	11.1%	10/465	2.2%	0/18	0.0%	148/465	31.8%
2009	1/11	9.1%	18/463	3.9%	1/11	9.0%	173/449	38.5%
2010	3/14	21.4%	58/451	12.9%	0/14	0.0%	157/451	34.8%
2011	0/9	0.0%	46/377	12.2%	0/9	0.0%	102/430	23.7%
2012	3/7	42.9%	135/475	28.4%	0/7	0.0%	217/447	48.5%

2013	1/6	16.7%	65/438	14.8%	3/6	50.0%	231/438	52.7%
2014	7/22	31.8%	135/432	31.3%	13/22	59.1%	277/432	64.1%
2015	8/24	33.3%	205/443	46.3%	10/22	45.5%	230/408	56.3%
2016	8/21	38.1%	266/479	55.5%	4/18	22.2%	289/465	62.1%

3.1.2 Univariate Regression

Individual linear and non-linear regression analyses conducted between *V. parahaemolyticus* concentration in oysters and eight measured variables from 2007-2016 identified water temperature, salinity, DO, pH, CHL and rainfall as significant model parameters in linear or non-linear regression. Model fit improved by less than 0.1 with log or log + 1 transformation of the independent variables. Water temperature accounted for the largest degree of *V. parahaemolyticus* variation (48.1%), DO accounted for 32.1%, followed by salinity (11.0%), pH (4.8%), CHL (2.8%) and rainfall (2.3%). The significance of pH increased in non-linear versus linear regression (Table 3), and the variability explained by pH also increased from 4.8 to 13.4%.

The form of the relationship between the environmental conditions and *V. parahaemolyticus* concentrations was further explored using loess smoothing to determine the parameters for each variable (Figure 5). The strength and significance of the linear response (Model 5) can be observed between *V. parahaemolyticus* and temperature, salinity and DO. Likewise, the non-linear relationship between pH and *V. parahaemolyticus,* identified by Model 6, is also highlighted by the loess smoothing. Though the fit between *V. parahaemolyticus* and CHL improved in Model 4 compared to Model 3, visual inspection of this relationship shows that this improvement can be attributed to rare events in the extremes of the observations.

Table 3. The relationship between *V. parahaemolyticus* concentrations and environmental variables and fit improvement based on linear (Model 5) and non-linear (Model 6) regression models in GBE in 2007-2017. Positive values indicate that the measure improved in Model 6 compared to Model 5 and negative values indicate a decrease in the model evaluation measurement.

Variable	Model 5	Model 6	Δ Model 6 – Model 5		
	p-value	p-value	Δ r^2	Δ Deviance	Δ AIC
Water Temperature (°C)	<0.001	<0.001	0.03	0.03	8.27
Dissolved Oxygen (mg/L)	<0.001	<0.001	0.04	0.05	7.28
Salinity (ppt)	<0.001	<0.001	-0.01	0.0	0.0
pH	0.009	0.002	0.14	0.08	8.48
Chlorophyll *a* (μg/L)	0.05	0.09	0.01	0.29	0.11
Rainfall (mm)	0.03	0.02	0.04	0.04	-6.31
Turbidity (NTU)	0.27	0.48	0.01	0.25	0.43
Total Dissolved Nitrogen (mg/L)	0.38	0.31	0.02	0.03	3.20

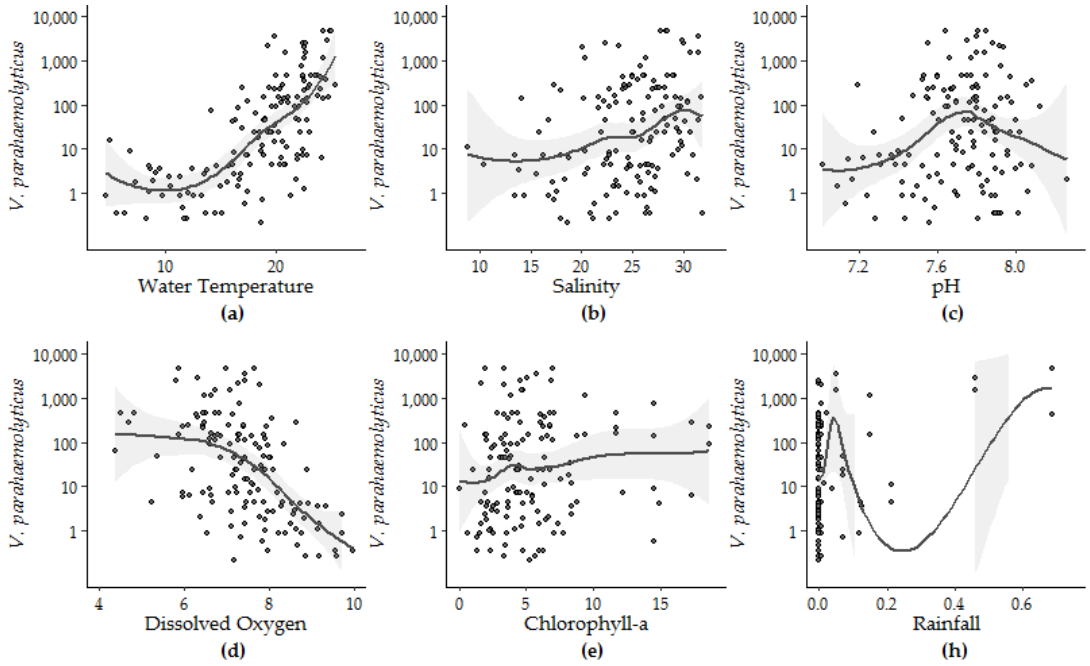


Figure 5. Loess smoothing applied to *V. parahaemolyticus* concentrations and **(a)** water temperature, **(b)** salinity, **(c)** pH, **(d)** DO - dissolved oxygen, **(e)** CHL - chlorophyll-*a*, and **(f)** rainfall.

The nonlinear regression between pH and *V. parahaemolyticus* was first improved with the addition of thin-plate splines. Based on the application of loess smoothing, pH was then re-parametrized as the square of the difference between the observed pH and 7.8, an apparent ecological optimum relative to observed *V. parahaemolyticus* concentrations in the study area. Re-parameterization of pH improved the percent variability explained, r^2 and p values from 4.8%, 0.04 and 0.008 (for the unmodified pH data) to 8.6%, 0.1 and 0.0003, respectively.

3.3 Sequential model building

A multiple regression model was next developed to determine a set of environmental variables that predict *V. parahaemolyticus* concentrations in oysters between 2007-2016 (Table 4). Water temperature was a foundational model variable for multiple regression model development and thus used in all multiple variable regression models. Single and multi-parameter models excluding water temperature explained less than the 48.1% of *V. parahaemolyticus* concentration variation explained by water temperature alone (data not shown). The addition of the trend term, photoperiod and harmonic regression variables to the environmental variables in negative binomial regression optimized model estimations (Figure 6, Table S2).

Table 4. The sequential building of multiple regression models for *V. parahaemolyticus* concentrations in oysters using GLM-G and GLM-NB models (Models 7, 8, 9).

Model composition [a]	Coefficients	St. Error	Deviance	AIC	Coefficients	St. Error	Deviance	AIC
Model 7 GLM-G					GLM-NB			
1. Temperature	0.34***	0.03	0.54	586.9	0.34***	0.03	0.48	1533.4
Salinity	0.12**	0.03			0.13***	0.03		
2. Temperature	0.37***	0.03	0.57	583.1	0.41***	0.03	0.51	1521.6

	C-pH	-4.73***	0.94			-5.52***	0.91		
3.	Temperature	0.35***	0.03			0.34***	0.02		
	C-pH	-3.93***	0.99	0.59	572.5	-4.38***	0.93	0.53	1518.3
	Salinity	0.07**	0.03			0.07**	0.02		
4.	**Temperature**	0.35***	0.02			0.34***	0.02		
	C-pH	4.61	3.35			5.52*	0.03		
	Salinity	0.11***	0.04	0.61	567.8	0.10**	2.97	0.57	1507.1
	C-pH*Salinity	-0.41***	0.16			-0.53**	0.14		
Model 8 GLM-G						GLM-NB			
1.	**Trend**	0.0003**	0.0001			0.0003***	0.0001		
	Photoperiod	-0.35**	0.11	0.62	564.4	-0.32***	0.09	0.58	1501.9
	Temperature	0.46***	0.04			0.43***	0.03		
	C-pH	-3.77***	0.95			-4.52***	0.86		
2.	Trend	0.0002**	0.001			0.0003***	0.0001		
	Photoperiod	-0.32**	0.11			-0.32***	0.13		
	Temperature	0.44***	0.04	0.62	565.9	0.43***	0.04	0.58	1503.9
	C-pH	-3.77***	0.99			-4.48***	0.89		
	Salinity	0.02	0.06			-0.004	0.03		
Model 9 GLM-G						GLM-NB			
1.	**Trend**	0.0003**	0.0001			0.0003***	0.0001		
	Sin(.)	0.07	0.69			-0.28	0.56		
	Cos(.)	1.47	1.12	0.62	566.3	0.79	0.91	0.58	1504.2
	Temperature	0.50***	0.11			0.41***	0.09		
	C-pH	-3.78***	0.96			-4.49***	0.87		
2.	Trend	0.0003**	0.0001			0.0003***	0.0001		
	Sin(.)	0.15	0.69			-0.31	0.57		
	Cos(.)	1.47	1.12	0.62	567.7	0.77	0.91	0.58	1506.2
	Temp	0.49***	0.11			0.41***	0.09		
	C-pH	-3.60***	0.99			-4.61***	0.89		
	Salinity	0.03	0.04			-0.006	0.03		

[a] significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1; [a] - pH data were treated as reparametrized C-pH variables

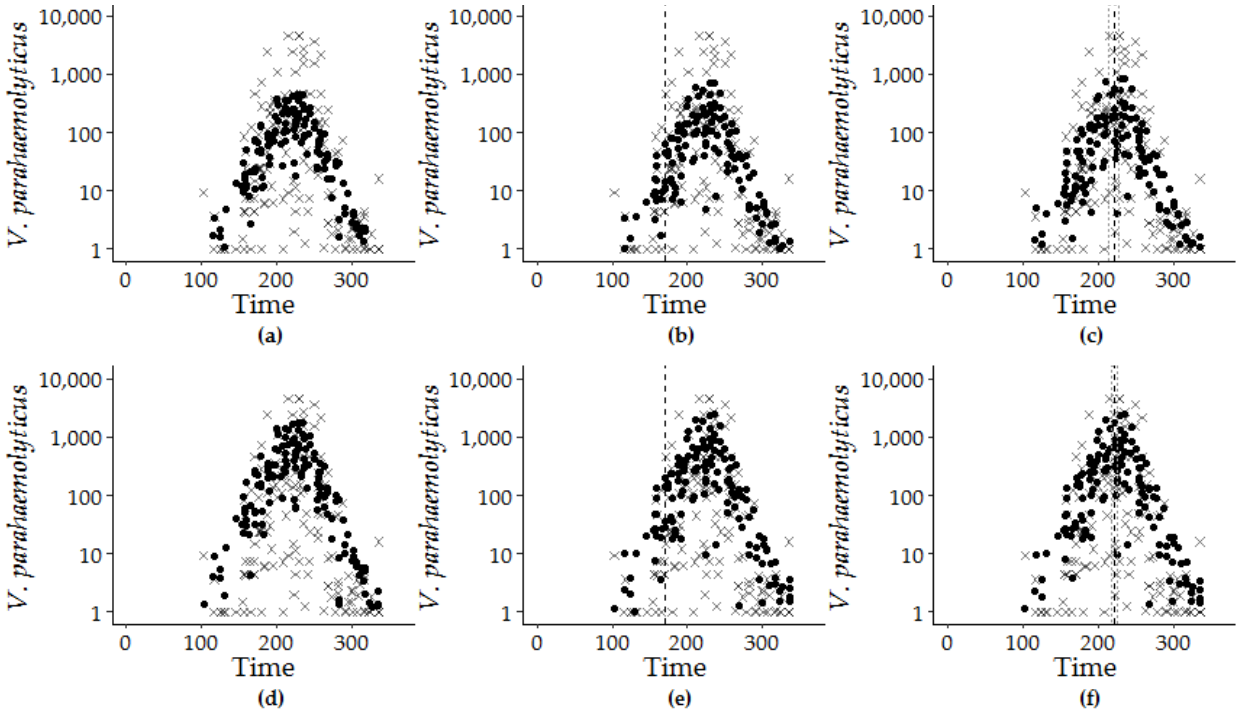


Figure 6. Model estimations (filled circle) and observed *V. parahaemolyticus* concentrations (x) are superimposed by the calendar day of the year from 2007-2016: GLM-G for **(a)** Model 7.4, **(b)** Model 8.1, **(c)** Model 9.1 and GLM-NB for **(d)** Model 7.4, **(e)** Model 8.1, and **(f)** Model 9.1. The dashed vertical line at day 170 for the hybrid model **(b)** and **(e)** marks the longest day of the year, and the dashed lines at day 222 ± 5 days and at day 221 ± 7 days indicate the calculated peak timing of *V. parahaemolyticus* concentration for Model 7.1 for **(c)** GLM-G and **(f)** GLM-NB versions.

Spearman rank correlation analysis of the individual intervals indicate that photoperiod, water temperature, DO, pH and salinity were significantly correlated with *V. parahaemolyticus* concentrations in all time intervals, though the correlation between pH and *V. parahaemolyticus* varied between intervals (Figure 7). Inter-variable correlations were observed between water temperature and DO ($R = -0.69$, $p < 0.0001$), salinity ($R = 0.20$, $p = 0.014$), CHL ($R = 0.21$, $p = <0.0001$) and rainfall ($R = 0.21$, $p = 0.015$). pH and DO ($R = 0.43$, $p <0.0001$) and pH and salinity ($R = 0.37$, $p <0.0001$) were also correlated. Rainfall was only significant in the test dataset whereas CHL was significant in the entire and training dataset (2007-13) intervals but not significant in the test dataset (2014-16) interval. Significant associations were observed between photoperiod and *V. parahaemolyticus* concentrations, water temperature, DO, salinity, CHL and pH ($R=0.28$, $p = <0.001$; $R=0.5$, $p = <0.001$; $R = -0.36$, $p = <0.001$, $R= 0.18$, $p = 0.04$; $R= 0.33$, $p = 0.001$; $R=-0.20$, $p =0.02$ respectively) (Figure 7). Of the nine variables considered, only turbidity, TDN and rainfall were not correlated to *V. parahaemolyticus* concentrations or photoperiod.

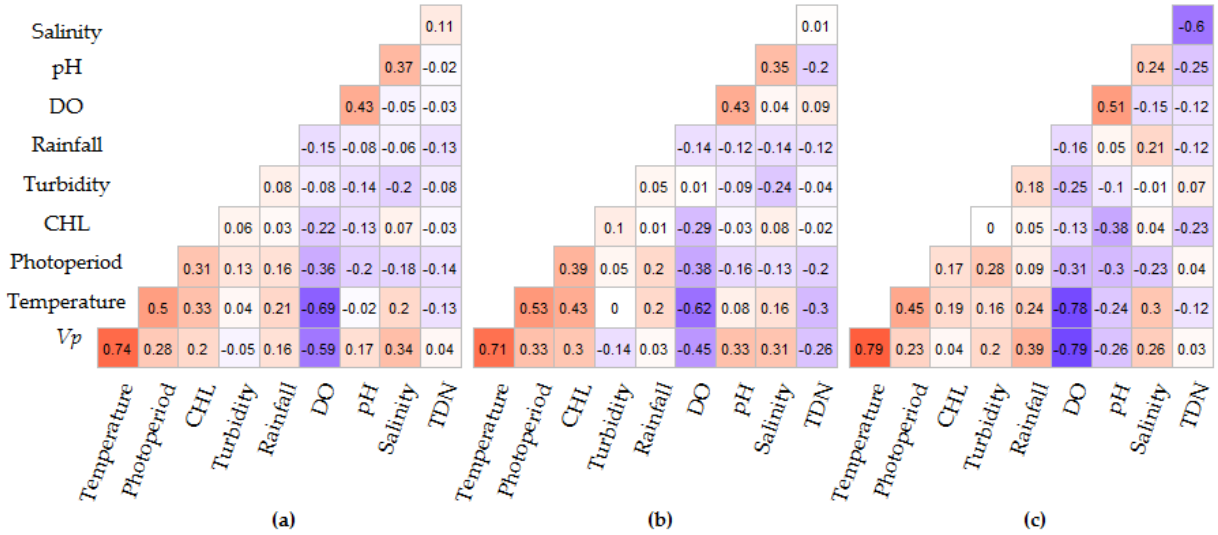


Figure 7. Spearman correlation analysis of *V. parahaemolyticus* concentrations and environmental variables for three intervals: **(a)** 2007-2016, **(b)** 2007-2013 and **(c)** 2014-2016. Red indicates positive and blue negative correlations and the degree of significance is highlighted by color intensity.

3.4 Model Performance-Prediction

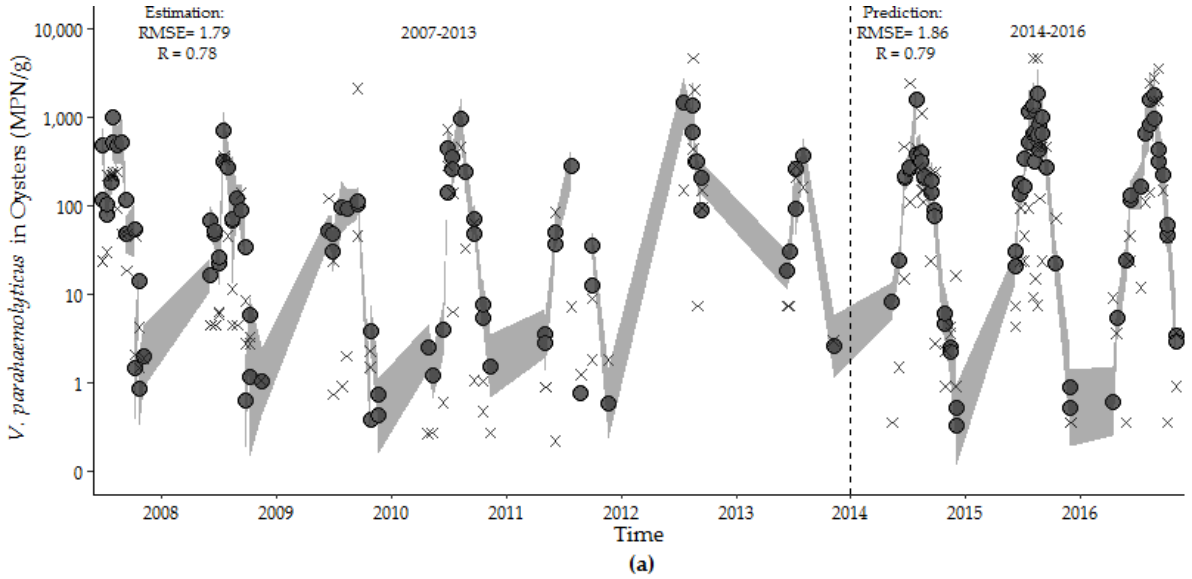
The hybrid model (Model 9.1) provided the best overall fit for each dataset time interval with consistently lower RMSE and higher r^2 values compared to the harmonic regression (Model 10.1) and environmental model (Model 7.4) (Table 5). The fits for all three models were relatively consistent even though the significance of some variables changed between time intervals. Although the estimations of precision for the harmonic regression model across training/test datasets were slightly lower than for other models, it is advantageous because important attributes of the data can be identified. For example, the *V. parahaemolyticus* concentrations peaked on 222 ± 5 day of the 365.25-day period for all three intervals. Similarly, the peak timing of water temperature and salinity were stable between the overall, training and test data sets (212 ± 2 day and 251 ± 18 day, respectively).

Table 5. The performance of three selected models: environmental model (Model 7.4), hybrid model (Model 8.1), and harmonic regression (Model 9.1) for three time periods: full (P1), training (P2), and testing (P3) intervals.

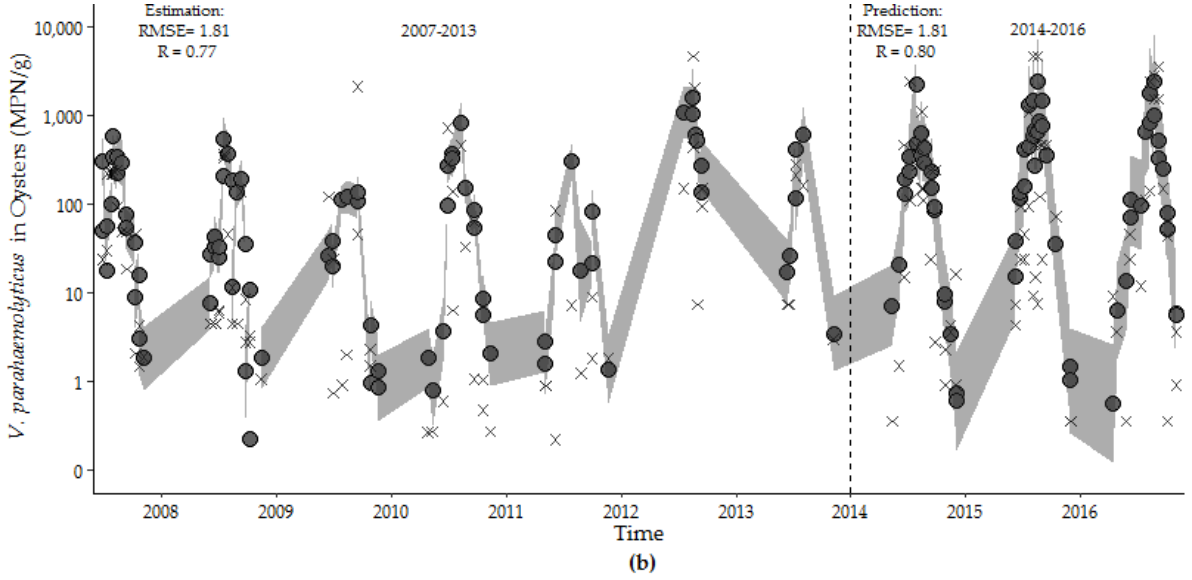
Model	Variable [a]		Time Interval		
			P1	P2	P3
Model 7.4	Coefficient:	Temperature	0.34***	0.37***	0.31***
		Salinity	0.10***	0.08**	0.24**
		C-pH	5.51*	5.12	266.01***
		Salinity*C-pH	-0.53***	-0.53***	-11.01***
	r^2		0.54	0.58	0.57
	Deviance		0.57	0.58	0.54
	RMSE		1.91	1.79	1.96
Model 8.1	Coefficient:	Trend	0.0003***	0.0003	0.0007
		Photoperiod	-0.31***	-0.28**	-0.48**
		Temperature	0.43***	0.45***	0.44***
		C-pH	-4.51***	-4.32***	-5.10

	r2		0.61	0.57	0.61
	Deviance		0.58	0.59	0.53
	RMSE		1.85	1.81	1.92
Model 9.1	Coefficient:	Trend	0.0004***	0.0004*	0.0008
		Sin(.)	-0.41	-1.88*	1.72*
		Cos(.)	0.63	-1.54	4.66**
		Temperature	0.40***	0.29**	0.74***
		C-pH	-4.30***	-4.20***	1.60
	r^2		0.61	0.55	0.63
	Deviance		0.58	0.60	0.54
	RMSE		1.81	1.82	1.83

[a] significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1.



(a)



(b)

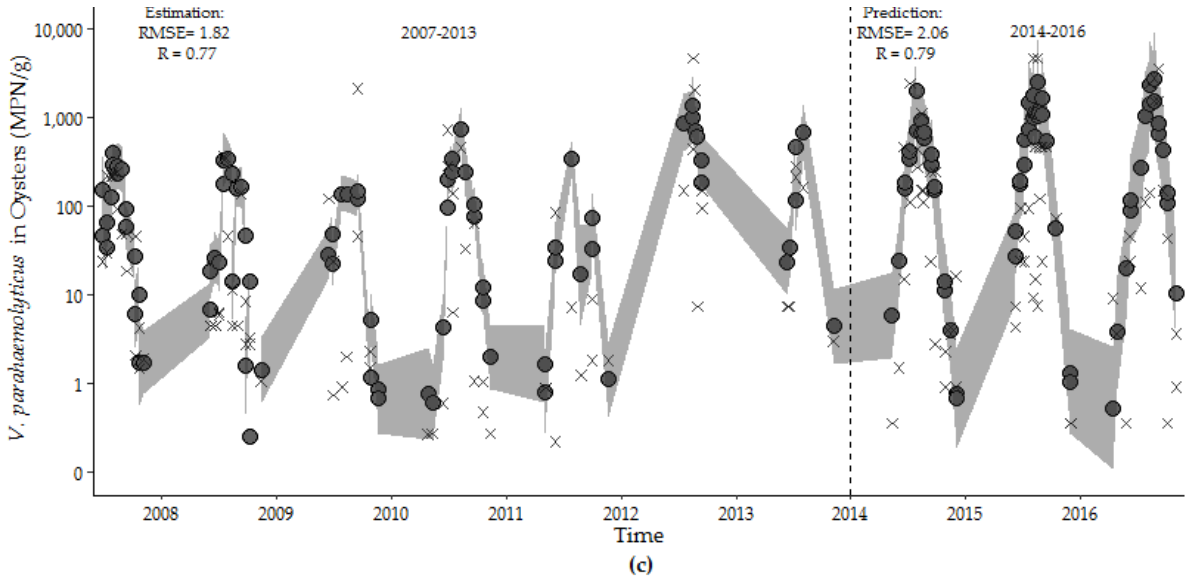


Figure 8. Estimates of *V. parahaemolyticus* concentrations (closed circle) with observed *V. parahaemolyticus* concentrations for: **(a)** environmental model, **(b)** hybrid model **(c)** harmonic regression models for training (2007-2013) and test (2014-2016) periods. The 95th percentile prediction interval is represented by the gray shading. Model fit values are shown in the upper left corner of each figure.

The environmental (Model 7.4), hybrid (Model 8.1,) and harmonic regression (Model 9.1) models developed with the observations from the training dataset accurately predict the overall trend, seasonality, and dispersion of the test dataset (Figure 8). The overall fits of all models were high, and the RMSE values increased in the short test time period. The hybrid model performed equally well in describing *V. parahaemolyticus* concentrations in the training dataset and predicting *V. parahaemolyticus* concentrations in the test dataset. This model contains a minimum number of environmental variables, photoperiod and calendar day of the study, and provides a good fit for capturing and predicting the seasonality, trend and dispersion of *V. parahaemolyticus* concentration during the study period.

4. Discussion

The intrinsic link that *V. parahaemolyticus* has with coastal ecosystems has been well studied and characterized. Previous studies have provided many useful site and time-specific descriptive models for describing *V. parahaemolyticus* concentration dynamics, however, few of them have been evaluated for their ability to forecast *V. parahaemolyticus* dynamics, or to be generalizable and transferable to other geographic areas or time periods. A wide range of environmental conditions and ecological interactions have been reported to influence, or at least correlate with, *V. parahaemolyticus* concentrations including water temperature, salinity, inorganic and organic nutrients, suspended solids-turbidity, chlorophyll-*a* and plankton levels, light availability, and meteorological conditions [4,5,16,17,27,29,36,38,51-61]. The temporal and spatial data analysis methods vary greatly in these studies, from simple correlation to more complex models [31]. These have often included application of multiple regression analysis to characterize and model the interactions between multiple environmental parameters and *V. parahaemolyticus* levels [5,18,28,36, 62, 63], even though they have not been useful for forecasting *V. parahaemolyticus* dynamics and risk conditions. Based on clearly observable aspects of the *V. parahaemolyticus* concentration data for this study and some initial analyses, the combination of models applied here incorporate seasonality, trend and dispersion concepts to characterize *V. parahaemolyticus* dynamics and accurately predict *V. parahaemolyticus* concentrations. Model accuracy is in part a function of using variables that are known and consistent such

as photoperiod or day of the year that are ecologically interpretable, but stable for effective *V. parahaemolyticus* forecasting. This approach of seasonality and trend analysis has the potential to be transferable for developing similar forecasting models patterns of *V. parahaemolyticus* dynamics in other locations.

V. parahaemolyticus concentrations in the GBE during this study followed the same pattern each year as concentrations increased rapidly each springtime as water temperatures increased, and after peak concentrations during the warmest summer conditions, decreased as water temperatures decreased in the fall each year. Such seasonality, where regular and predictable changes in environmental and climatic conditions re-occur every calendar year, tends to become more pronounced with increasing distance from the equator and is largely due to extreme temperature variation driven by variable photoperiod [64]. Water temperature accounted for approximately 48.1% of the variation observed in *V. parahaemolyticus* concentrations in this study, similar to what has been observed globally and especially in highly seasonal, temperate water regions [27, 28, 59]. Thus, seasonality is a significant aspect of *V. parahaemolyticus* concentration dynamics in temperate coastal areas like New Hampshire and the Northeast US.

Photoperiod and harmonic regression models along with correlation analysis showed that *V. parahaemolyticus* concentration, water temperature, dissolved oxygen, pH, salinity and chlorophyll-*a* are significantly related to variables that mirror seasonal patterns in the GBE. Likewise, these variables accurately estimate *V. parahaemolyticus* concentrations in oysters. The synchronized seasonal periodic oscillation is one probable explanation for why regression modeling favors water temperature as the most significant model parameter. A complex combination of biological and physical environmental variables certainly drives *V. parahaemolyticus* population dynamics, however, many of these environmental variables are, in turn, driven mainly by seasonal temperature. Therefore, the variability they contribute to *V. parahaemolyticus* concentrations is not significantly different than what is provided by water temperature. For example, dissolved oxygen was negatively correlated with *V. parahaemolyticus* concentrations, similar to what has been previously reported [20] and was the second strongest variable, estimating over 32% of the variability in *V. parahaemolyticus* over the course of the study in the GBE. Since *V. parahaemolyticus* is a facultative anaerobe, this finding has the potential to elucidate important biological dimensions of the ecology of *V. parahaemolyticus*. Water temperature is a dominant driver of dissolved oxygen concentrations so collinearity between these variables is likely. In addition, because of the constraints of mathematical modeling, well-fit models are not necessarily mechanistically or ecologically descriptive [65], and in this case, dissolved oxygen was omitted from model development to avoid multicollinearity in favor of water temperature as a stronger model variable.

Salinity and water temperature are both seasonally variable parameters that, together are the most commonly cited environmental drivers of *V. parahaemolyticus* concentration variation [16, 31, 66]. Salinity was a significant predictive parameter for *V. parahaemolyticus* concentration in this study, though the significance of salinity was dependent on the time interval (2007-2013 versus 2014-2016) of the data and the trend adjustment in the model (Table S2). This type of variability has also been observed in risk assessment [67, 68] and in previous studies where salinity sometimes shows a strong positive correlation with *V. parahaemolyticus* [5,6,25, 62], whereas for others [28, 52, 59, 69], salinity and *V. parahaemolyticus* dynamics do not correlate. Thus, the finding that salinity and other variables reported to be significant in other *V. parahaemolyticus* concentration models were not included in this study's final model may be, at least in part, a function of both the specific conditions at this study site and time period and a function of the in-depth statistical approach used.

Though most studies find little to no correlation between pH and *V. parahaemolyticus* concentration [20,21,28], non-linear regression and correlation analysis identified pH as an important parameter for the predictive models in the GBE. Loess smoothing highlighted the marked non-linearity of the relationship between pH and *V. parahaemolyticus* concentrations and suggested a biological optimum/optimal range for pH where *V. parahaemolyticus* concentrations decreased as pH increased or decreased relative to pH 7.8. For

the purposes of optimal model development, a new pH variable was constructed by reparametrizing the measurements to create a linear response in *V. parahaemolyticus* as pH measurements moved from the optima of 7.8. An optimal pH of 7.8 is near the pH (8.5) of alkaline peptone water medium used to optimally enrich for *Vibrio* species [41] and has also been suggested as an optimal pH by laboratory-based observations [70]. Wong et al., (1998) [71] found that exposure to more acidic environments tended to reduce cell density and cause stress responses in *V. parahaemolyticus*. In this study, we observe that pH measurements in the GBE appeared to become less variable and more basic in recent years, which was also reported by Lopez-Hernandez et al., (2015)[5]. Thus, going beyond simple linear regression and including the use of non-linear analysis reveals pH as an important and ecologically linked variable to explain *V. parahaemolyticus* population dynamics.

In other studies [36,59, 62], variables other than salinity and pH were significant for estimating *V. parahaemolyticus* concentrations in univariate regression, however in this study they provided an insignificant amount of improvement to a multiple regression model that included water temperature. For example, chlorophyll-*a*, considered a proxy measurement for phytoplankton abundance [21,31], was significantly related to *V. parahaemolyticus* concentrations in correlation and univariate regression, but it was not significant in a multiple regression model that included water temperature. Chlorophyll-*a* was thus omitted from further model development because it did not contribute additional information in describing *V. parahaemolyticus* variation. Many studies have suggested an important ecological interaction between *V. parahaemolyticus* and plankton [27,54, 56, 62,68,72,73], and though chlorophyll-*a* was not included in the multiple regression models, we have also conducted a parallel study to explore the relationship between *V. parahaemolyticus* and plankton species across several years in the GBE [74,75] to determine covarying plankton species. These have included phytoplankton that have been reported to be significantly associated with *V. parahaemolyticus* elsewhere [76,77] that could provide more in-depth insight into the importance of phytoplankton and the proxy chlorophyll-*a* to the *V. parahaemolyticus* concentration dynamics observed in the GBE.

Approximately half of the variability of *V. parahaemolyticus* in the GBE could be predicted using the contribution of photoperiod (in hours), sine and cosine of the day of the study in harmonic regression, and the day of the study. Even though the model consisting solely of environmental variables was potentially more ecologically informative, the trend and seasonality variables of calendar day of the study, photoperiod, sine and cosine were more stable to estimate and predict the patterns of seasonality and trend of increasingly high concentrations over time in *V. parahaemolyticus* than salinity and to a lesser degree pH and do not require in situ measurements. Additionally, evaluation of the environmental model for its forecasting ability highlighted that some evaluation measures were discordant, whereas the harmonic regression and photoperiod model goodness-of-fit and forecasting error were in agreement. This highlights that though multiple evaluation measures can cause complexities in model selection, in this study the model with conflicting evaluation measures may indicate underlying issues, whereas the models where evaluation measurements were in agreement provided stronger prediction accuracy. Harmonic regression analyses also lead to identification of the day of year for peak *V. parahaemolyticus* concentration that occurs in mid-August (day 222 ± 5 days) that followed the peak timing of water temperature (213 ± 2), whereas the longest day of the year is June 21st (day 170). This highlights a loading, or hysteresis in the system and provides the basis for understanding the 'fall shoulder' of elevated concentrations of *V. parahaemolyticus* that extend into the late September.

Peak timing was used to assess each environmental variable individually to detect how environmental variables may contribute to the development of ideal conditions for *V. parahaemolyticus*. Data in this study were collected either monthly or biweekly, whereas *V. parahaemolyticus* replicates every eight minutes under ideal conditions. In this instance, accurate detection of lagged effects on *V. parahaemolyticus* would require more frequent sampling and fine-scale temporal resolution. Due to this level of biological complexity and the irregular temporal intervals of the data in our study, the mean from 12 hours

proceeding collection was used for regression with environmental variables and peak timing was used to assess temporally how each environmental variable may contribute to the development of ideal conditions for *V. parahaemolyticus*. Using this approach, we determined that significant predictive variables peak in advance of *V. parahaemolyticus* potentially contributing to a hysteresis or loading of the systems, setting up conditions that are optimal for *V. parahaemolyticus*. Davis et al., 2019 [78] recently reported that environmental variables approximately one month proceeding collection were significant to predicting *V. parahaemolyticus* concentrations in the Chesapeake Bay suggesting they might also be observing this type of lagged effect from a loading of the system. The application of harmonic regression and peak timing here demonstrates how biological complexities and limitations of sampling frequency necessary can be overcome whereas also providing the resolution to detect temporal patterns between dependent and independent variables. The determination of peak timing is also a potentially important tool for forecasting the commonly observed mid-summer peaks in illnesses in the Northeast US [79].

A major characteristic of the *V. parahaemolyticus* concentration data is its wide dispersion. The comparison between Gaussian and negative binomial GLMs determined that the dispersion of *V. parahaemolyticus* concentrations, especially the extreme high concentrations, was best fit by the negative binomial model, as it can better account for the wide range of *V. parahaemolyticus* concentrations (0.3 to 4600 MPN/g) observed annually in the GBE. Effective risk models, with negative binomial regression as an essential model attribute, developed to predict the increasing and more dispersed *V. parahaemolyticus* concentrations will become more important as global warming and other climate and ecosystem changes will probably cause increased concentrations and persistence of *V. parahaemolyticus* in temperate coastal areas [8,80-82] with a likely increase in public health risks.

Model evaluation, estimations, and predictions illustrate how each model provides fit and prediction ability of the variability in *V. parahaemolyticus* concentration observed over the course of the study. Though a forecasting model consisting of environmental variables could be more appealing because of its ecological interpretability, there are potential limitations to models that rely solely on environmental predictors. For example, it is unlikely that a well-fit model can contain all the environmental variables that effect *V. parahaemolyticus* given its ecological complexity and the collinearity between seasonal-driven variables that relate to *V. parahaemolyticus* dynamics. Further, the strength of environmental variables to predict *V. parahaemolyticus* over time can change, as was observed in the interaction between pH and salinity between time intervals. Additionally, salinity became insignificant when the model was adjusted for a linear trend. The negative binomial harmonic regression and hybrid models fit the seasonality and trend features, and account well for dispersion of *V. parahaemolyticus.* All models demonstrated good forecasting ability. Importantly, these models also enabled the determination of key characteristics of *V. parahaemolyticus* in the GBE including peak timing and a seasonal loading contributing to prolonged elevated concentrations that last into fall months. The hybrid model provides the optimal level of ecological interpretability, a reasonable ability to capture the dynamics of *V. parahaemolyticus* concentrations in oysters in the GBE, and offers a stable platform for forecasting *V. parahaemolyticus* concentrations in coming seasons. Thus, the use of both significant environmental variables and stable parameters in the hybrid negative binomial regression model lead to successful forecasting model development that captures seasonality, temporal trends, and the high degree of data variability and dispersion.

The increased incidence of illnesses caused by *V. parahaemolyticus* infections in the Northeast US has co-occurred with increases in regional surface water temperatures and other environmental parameters, as shown in this study, suggesting an increase in the presence of pathogenic *V. parahaemolyticus* strains and/or population evolution [13,14]. The model approach developed in this study illustrates how characteristics of *V. parahaemolyticus* dynamics can be captured as environmental conditions continue to become more favorable for the pathogen to enable accurate prediction of public health risk to shellfish consumers and recreational users of coastal waters. This information, coupled with recent advances [13,14,19] that improve

detection methods for endemic and invasive pathogenic *V. parahaemolyticus* sequence types (ST) in the Northeast, could be useful for shellfish harvest management in the Northeast US based on this new improved and integrated capacity to forecast concentration dynamics of both total and pathogenic *V. parahaemolyticus* populations and potential disease outbreak risks. The developed modeling approach also has the potential to inform more in-depth mechanistic studies to gain a better understanding of the ecology of *V. parahaemolyticus* and other water-borne pathogens.

5. Conclusions

This study suggests that transferable models can be developed for forecasting public health risks related to *V. parahaemolyticus* concentrations in shellfish. Ecological monitoring data and statistical modeling are necessary to effectively characterize relationships between ecological variables and *V. parahaemolyticus* concentrations. From among many ecological variables, easy to measure water temperature and pH were all that was required when combined with seasonality and trend variables within hybrid statistical models to capture both long-term increasing trends for *V. parahaemolyticus* concentrations and to provide capacity for forecasting *V. parahaemolyticus* concentrations. The determination of peak timing is useful for assessing how each environmental variable may contribute to the development of optimal conditions for *V. parahaemolyticus*. This approach may be best applied in temperate, seasonally driven regions like the Northeast, US, as it relies on characteristics of *V. parahaemolyticus* ecology that are shared by most temperate regions.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Trend and seasonality estimates detected by Model 3 and Model 4. Table S2: Stepwise Development of Model 6 and 7.

Author Contributions: Conceptualization, MAH. EAU, CAW, VSC, ENN and SHJ.; methodology, MAH, EAU, CAW, VSC, ENN and SHJ.; software, MAH, EAU.; validation, CAW, VSC, ENN and SHJ.; formal analysis, MAH and EAU; resources, CAW, VSC, SHJ.; data curation, MAH and EAU.; writing—original MAH, EAU; writing—review and editing, CAW, VSC, ENN and SHJ.; visualization, MAH and EAU.; supervision, SJH.; funding acquisition, CAW, VSC, SHJ.

Funding: The authors gratefully acknowledge partial funding support from the National Science Foundation EPSCoR IIA-1330641, USDA National Institute of Food and Agriculture Hatch NH00574, NH00609 (accession 233555), and NH00625 (accession 1004199), and the National Oceanic and Atmospheric Administration College Sea Grant program and New Hampshire Sea Grant program grants R/CE-137, R/SSS-2, R/HCE- 3 and in part funding support from NSF IRES Track I: Collaborative Research: U.S.-Indonesian Research Experience for Students on Sustainable Adaptation of Coastal Areas to Environmental Change (award #1826939, PI Naumova).

Acknowledgments: The authors would like to thank Jennifer Mahoney, Meg Striplin, Brian Schuster, Crystal Ellis, Jong Yu, Eliot Jones, Michael Taylor, Ashley Marcinkiewicz, Feng Xu, Tom Gregory, Chris Peters, Jackie Lemaire, Audrey Berenson, Sarah Richards, Emily Schulz, and Elizabeth Deyett for their help with sampling, sample processing, detection analysis, and database management. Also, we thank Dr. Iago Hale, Alexandra Kulinkina and Tania M. Alarcon Falconi for support with implementing harmonic regression analysis, peak timing calculations in the R environment, and other statistical approaches.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

CDC. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data). Atlanta, Georgia: U.S. Department of Health and Human Services, CDC. 2017.

Nilsson, W. B., Paranjpye, R. N., Hamel, O. S., Hard, C., & Strom, M. S. Vibrio parahaemolyticus risk assessment in the Pacific Northwest: it's not what's in the water. *FEMS microbiology ecology* **2019**, 95(4), fiz027..

Newton, A.; Garrett, N.; Stroika, S.; Halpin, J.; Turnsek, M.; Mody, R. Notes from the field: increase in *Vibrio parahaemolyticus* infections associated with consumption of Atlantic coast shellfish-2013. *Morbidity and Mortality Weekly Report (MMWR)* **2014**, *(Vol. 63).*

Ceccarelli, D.; Hasan, N. A.; Huq, A.; Colwell, R. R. Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Frontiers in cellular and infection microbiology* **2013**, *3*, 97.

López-Hernández, K.M.; Pardío-Sedas, V.T.; Lizárraga-Partida, L.; Williams, J.D.J.; Martínez-Herrera, D.; Flores-Primo, A.; Uscanga-Serrano, R.; Rendón-Castro, K. Environmental parameters influence on the dynamics of total and pathogenic *Vibrio parahaemolyticus* densities in *Crassostrea virginica* harvested from Mexico's Gulf coast. *Marine pollution bulletin* **2015**, *91(1),* pp.317-329.

Vezzulli, L., Colwell, R. R., & Pruzzo, C. Ocean warming and spread of pathogenic *Vibrios* in the aquatic environment. *Microbial ecology* **2013**, 65(4), 817-825.

Baker-Austin, C.; Trinanes, J. A.; Taylor, N. G.; Hartnell, R., Siitonen, A.; Martinez-Urtaza, J. Emerging Vibrio risk at high latitudes in response to ocean warming. *Nature Climate Change* **2013**, *3(1),* 73.

Baker-Austin, C.; Trinanes, J.; Gonzalez-Escalona, N.; Martinez-Urtaza, J. Non-cholera vibrios: the microbial barometer of climate change. *Trends in microbiology* **2017,** *25(1)*, 76-84.

Centers for Disease Control and Prevention (CDC). (2013). Increase in *Vibrio parahaemolyticus* illnesses associated with consumption of shellfish from several Atlantic coast harvest areas, United States, 2013. *Vibrio Illness (Vibriosis)* **2013**, *21*.

Jones, S.H. (2011). Microbial Pathogens and Biotoxins: State of the Gulf of Maine Report. Gulf of Maine Council on the Marine Environment. http://www.gulfofmaine.org/2/sogom-homepage/. 21 pp.

Martinez-Urtaza, J.; Baker-Austin, C.; Jones, J.; Newton, A.; DePaola, A. Spread of Pacific Northwest *Vibrio parahaemolyticus* strain. *New England Journal of Medicine* **2013**, *369*(1573-1574).

McLaughlin, J.B.; DePaola, A.; Bopp, C.A.; Martinek, K.A.; Napolilli, N.P.; Allison, C.G.; Murray, S.L.; Thompson, E.C.; Bird, M.M.; Middaugh, J.P. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. *New England Journal of Medicine* **2005**, *353*(14), pp.1463-1470.

Xu, F.; Ilyas, S.; Hall, J. A.; Jones, S. H.; Cooper, V. S.; Whistler, C. A. Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages. *Frontiers in Microbiology*, **2015**, *6*, 272.

Xu, F.; Gonzalez-Escalona, N.; Drees, K. P.; Sebra, R. P.; Cooper, V. S.; Jones, S. H.; Whistler, C. A. Parallel evolution of two clades of an Atlantic-endemic pathogenic lineage of *Vibrio parahaemolyticus* by independent acquisition of related pathogenicity islands. *Appl. Environ. Microbiol*. **2017**, *83(18*), e01168-17.

Lipp, E. K.; Huq, A.; Colwell, R. R. Effects of global climate on infectious disease: the cholera model. *Clinical microbiology reviews* **2002**, *15*(4), 757-770.

Lovell, C. R. Ecological fitness and virulence features of Vibrio parahaemolyticus in estuarine environments. *Applied microbiology and biotechnology* **2017**, 101(5), 1781-1794.

Martinez-Urtaza, J.; Blanco-Abad, V.; Rodriguez-Castro, A.; Ansede-Bermejo, J.; Miranda, A.; Rodriguez-Alvarez, M. X.; Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus in* offshore areas. *The ISME Journal* **2012**, *6*(5), 994–1006.

Urquhart, E.A.; Zaitchik, B.F.; Guikema, S.D.; Haley, B.J.; Taviani, E.; Chen, A.; Brown, M.E.; Huq, A.; Colwell, R.R., 2016. Use of Environmental Parameters to Model Pathogenic Vibrios in Chesapeake Bay. *Journal of Environmental Informatics* **2016,** *26*(1), 1-13.

Whistler, C. A.; Hall, J. A.; Xu, F.; Ilyas, S.; Siwakoti, P.; Cooper, V. S.; Jones, S. H. Use of whole-genome phylogeny and comparisons for development of a multiplex PCR assay to identify sequence type 36 *Vibrio parahaemolyticus. Journal of clinical microbiology* **2015**, *53(6*), 1864-1872.

Blackwell, K. D.; Oliver, J. D. The ecology of *Vibrio vulnificus, Vibrio cholerae, and Vibrio parahaemolyticus* in North Carolina estuaries. *The Journal of Microbiology* **2008,** *46(2),* 146-153.

Caburlotto, G.; Haley, B. J.; Lleò, M. M.; Huq, A.; Colwell, R. R. Serodiversity and ecological distribution of *Vibrio parahaemolyticus* in the Venetian Lagoon, Northeast Italy. *Environmental microbiology reports* **2010,** *2(1*), 151-157.

Cook, D. W.; Bowers, J. C.; DePaola, A. Density of total and pathogenic (*tdh*+) *Vibrio parahaemolyticus* in Atlantic and Gulf Coast molluscan shellfish at harvest. *Journal of food protection* **2002,** *65*(12), 1873-1880.

Froelich, B. A.; Ayrapetyan, M.; Fowler, P.; Oliver, J. D.; Noble, R. T. Development of a matrix tool for the prediction of Vibrio species in oysters harvested from North Carolina. *Applied and Environmental Microbiology* **2015**, *81*(3), 1111–9.

Johnson, C. N.; Flowers, A. R.; Noriea, N. F.; Zimmerman, A. M.; Bowers, J. C.; DePaola, A.; Grimes, D. J. Relationships between environmental factors and pathogenic vibrios in the northern Gulf of Mexico. *Appl. Environ. Microbiol.*, **2010,** *76*(21), 7076-7084.

Johnson, C.N.; Bowers, J.C.; Griffitt, K.J.; Molina, V.; Clostio, R.W.; Pei, S.; Laws, E.; Paranjpye, R.N.; Strom, M.S.; Chen, A; Hasan, N.A. Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). *Appl. Environ. Microbiol.* **2012**, *78*(20), pp.7249-7257.

Julie, D.; Solen, L.; Antoine, V. Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll *a*. *Environmental Microbiology* **2010,** *12*(4), 929–937.

Kaneko, T., & Colwell, R. R. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. *Journal of Bacteriology* **1973,** *113(1*), 24-32.

Paranjpye, R. N.; Nilsson, W. B.; Liermann, M.; Hilborn, E.D.; George, B.J.; Li, Q.; Bill, B.D.; Trainer, V.L.; Strom, M.S.; Sandifer, P.A. Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA. *FEMS microbiology ecology* **2015,** *91(12),* 121.

Parveen, S.; Hettiarachchi, K.A.; Bowers, J.C.; Jones, J.L.; Tamplin, M.L.; McKay, R.; Beatty, W.; Brohawn, K.; DaSilva, L.V.; DePaola, A. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International journal of food microbiology* **2008**, *128*(2), 354-361.

Parveen, S.; DaSilva, L.; DePaola, A.; Bowers, J.; White, C.; Munasinghe, K.A.; Brohawn, K.; Mudoh, M.; Tamplin, M.L. Development and validation of a predictive model for the growth of *Vibrio parahaemolyticus* in post-harvest shellstock oysters. *Int. J. Microbiol*. **2013,** *161*:1-6

Takemura, A.; Chien, D.; Polz, M. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology* **2014**, *5*(38).

Froelich, B. A.; Noble, R. T. Vibrio bacteria in raw oysters: managing risks to human health. *Philosophical Transactions of the Royal Society B: Biological Sciences* **2016,** *371*(1689), 20150209.

Crystal, E. N.; Schuster, B.M.; Striplin, M.J.; Jones, S.H.; Whistler, C.A.; Cooper, V.S. Influence of seasonality on the genetic diversity of *Vibrio parahaemolyticus* in New Hampshire shellfish waters as determined by multilocus sequence analysis. *Appl. Environ. Microbiol*. **2012**, *78(10*), 3778-3782.

Mahoney, J. C.; Gerding, M.J.; Jones, S.H.; Whistler, C.A. Comparison of the pathogenic potentials of environmental and clinical *Vibrio parahaemolyticus* strains indicates a role for temperature regulation in virulence. *Appl. Environ. Microbiol*. **2010**, *76(22),* 7459-7465.

Schuster, B.M.; Tyzik, A.L.; Donner, R.A.; Striplin, M.J.; Almagro-Moreno, S.; Jones, S.H.; Cooper, V.S.; Whistler, C.A. Ecology and genetic structure of a northern temperate *Vibrio cholerae* population related to toxigenic isolates. *Appl. Environ. Microbiol.* **2011**, *77(21*), 7568-7575.

Urquhart, E. A.; Jones, S. H.; Jong, W. Y.; Schuster, B. M.; Marcinkiewicz, A. L.; Whistler, C. A.; Cooper, V. S. Environmental conditions associated with elevated *Vibrio parahaemolyticus* concentrations in Great Bay Estuary, New Hampshire. *PloS one* **2016**, *11*(5), e0155018.

Bartley, C. H.; Slanetz, L. W. Occurrence of *Vibrio parahaemolyticus* in Estuarine Waters and Oysters of New Hampshire. *Applied and Environmental Microbiology* **1971,** *21*(5), 965–966.

Jones, S.; Summer-Brason, B. Incidence and detection of pathogenic *Vibrio* spp. in a northern New England estuary, USA. *Journal of Shellfish Research* **1998,** *17*, 1665–1669.

O'Neill, K.; Jones, S.; Grimes, D. Incidence of *Vibrio vulnificus* in northern New England water and shellfish. *FEMS Microbiology Letters* **1990,** *72(1-2*), 163–167.

Nash, C.; B. Dejadon. (2019). New Hampshire *Vibrio parahaemolyticus* Risk Evaluation. New Hampshire Department of Environmental Services, Shellfish Program, Portsmouth, NH.

Kaysner, C.; DePaola, A. (2004) Chapter 9: *Vibrio*. In:BAM Council (eds) Bacteriological analytical manual online. US Food and Drug Administration. Available at https://www.fda.gov/food/laboratory-methods-food/bam-vibrio (accessed June 17, 2019).

Panicker, G.; Call, D. R.; Krug, M. J.; Bej, A. K. Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Applied and Environmental Microbiology* **2004**, *70*(12), 7436–44.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Faraway, J. J. (2016). Extending the linear model with R: generalized linear, mixed effects and nonparametric regression models. Chapman and Hall/CRC.

Hastie, T., & Tibshirani, R. (1990). *Generalized Additive Models* (Vol. 43). CRC Press.

Wood, S.N. (2017) Generalized Additive Models: An Introduction with R (2nd edition).Chapman and Hall/CRC.

Ruckstuhl, A. (2010). Introduction to nonlinear regression. *IDP Institut fur Datenanalyse und Prozessdesign, Zurcher Hochschule fur Angewandte Wissenschaften. See: http://www. idp. zhaw. ch.(Cited on p. 365.)*.

Akaike, H. (1974). A new look at the statistical model identification. In *Selected Papers of Hirotugu Akaike* (pp. 215-222). Springer, New York, NY.

Naumova, E.N.; MacNeill, I.B. Seasonality Assessment for Biosurveillance. In: *Advances in Statistical Methods for the Health Sciences.* Auget JL, Balakrishnan N, Mesbah M, Molenberghs G, eds.. Birkhauser. **2007**. pp437–450.

Alarcon Falconi, T.M.; Cruz, M.; Naumova, E.N. The shift in seasonality of legionellosis in the U.S. *Epidemiol Infect*. **2018**, *146*, 1824-1833.

Cox, A.; Gomez-Chiarri, M. *Vibrio parahaemolyticus* in Rhode Island coastal ponds and the estuarine environment of Narragansett Bay. *Applied and Environmental Microbiology* **2012;** *78*(8), 2996–2999.

DePaola, A.; Hopkins, L.H.; Peeler, J.T.; Wentz, B.; McPhearson, R.M. *Incidence of Vibrio parahaemolyticus* in US coastal waters and oysters. *Appl. Environ. Microbiol*. **1990,** *1;56(8*), 2299-302.

Deepanjali, A.; Kumar, H. S.; Karunasagar, I. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. *Appl. Environ. Microbiol*. **2005,** 71(7), 3575-3580.

Kaneko, T.; Colwell, R. R. The annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. *Microbial Ecology* **1997**, *4(2),* 135-155.

Lovell, C.R. Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Applied microbiology and biotechnology* **2017**, 101(5),1781-94.

Oberbeckmann, S.; Wichels, A.; Wiltshire, K. H.; Gerdts, G. Occurrence of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in the German Bight over a seasonal cycle. *Antonie Van Leeuwenhoek* **2011**, *100(2)*, 291-307.

Su, Y. C.; Liu, C. *Vibrio parahaemolyticus*: a concern of seafood safety. *Food microbiology* **2007**, *24*(6), 549-558.

Turner, J. W.; Malayil, L.; Guadagnoli, D.; Cole, D.; Lipp, E. K. Detection of *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae* with respect to seasonal fluctuations in temperature and plankton abundance. *Environmental microbiology* **2014,** 16(4), 1019-1028.

Oberbeckmann, S.; Fuchs, B. M.; Meiners, M.; Wichels, A.; Wiltshire, K. H.; Gerdts, G. Seasonal dynamics and modeling of a Vibrio community in coastal waters of the North Sea. *Microbial ecology* **2012,** *63*(3), 543-551.

Davis, B. J.; Jacobs, J. M.; Davis, M. F.; Schwab, K. J.; DePaola, A.; Curriero, F. C. Environmental determinants of *Vibrio parahaemolyticus* in the Chesapeake Bay. *Appl. Environ. Microbiol.* **2017,** *83*(21), e01147-17.

Vezzulli, L.; Pezzati, E.; Moreno, M.; Fabiano, M.; Pane, L.; Pruzzo, C.; VibrioSea Consortium. Benthic ecology of *Vibrio* spp. and pathogenic Vibrio species in a coastal Mediterranean environment (La Spezia Gulf, Italy). *Microbial ecology* **2009,** *58*(4), 808-818.

Froelich, B.; Bowen, J.; Gonzalez, R.; Snedeker, A.; Noble, R. Mechanistic and statistical models of total Vibrio abundance in the Neuse River Estuary. *Water research*, **2013**, *47(15),* 5783-5793

Hsieh, J.; Fries, J.; Noble, R. Dynamics and predictive modelling of *Vibrio* spp. in the Neuse River Estuary, North Carolina, USA. *Environmental Microbiology* **2008,** *10*(1), 57–64.

Tonkin, J. D.; Bogan, M. T.; Bonada, N.; Rios-Touma, B.; Lytle, D. A. Seasonality and predictability shape temporal species diversity. *Ecology* **2017,** *98*(5), 1201-1216.

Shmueli, G. To explain or to predict? *Statistical science* **2010**, 25(3), 289-310.

DePaola, A.; Nordstrom, J.L.; Bowers, J.C.; Wells, J.G.; Cook, D.W. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Appl. Environ. Microbiol*. **2003**, 69:1521–1526.

Young, I.; Gropp, K..; Fazil, A.; Smith, B. A. Knowledge synthesis to support risk assessment of climate change impacts on food and water safety: A case study of the effects of water temperature and salinity on *Vibrio parahaemolyticus* in raw oysters and harvest waters. *Food Research International* **2015**, *68*, 86-93.

Parveen, S., Jahncke, M., Elmahdi, S., Crocker H., Bowers, J., White, C., Gray, S., Morris, A.C., & Brohawn, K. High salinity relaying to reduce Vibrio parahaemolyticus and Vibrio vulnificus in Chesapeake Bay oysters (Crassostrea virginica). *Journal of food science* **2017**, 82, no. 2: 484-491.

Turner, J. W.; Good, B.; Cole, D.; Lipp, E. K. Plankton composition and environmental factors contribute to Vibrio seasonality. *The ISME journal* **2009**, *3(9*), 1082.

Wong, H. C.; Wang, P. Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of applied microbiology* **2004**, 96(2), 359-366.

Wong, H. C.; Peng, P. Y.; Han, J. M.; Chang, C. Y.; Lan, S. L. Effect of mild acid treatment on the survival, enteropathogenicity, and protein production in *Vibrio parahaemolyticus*. *Infection and immunity* **2015**, *66*(7), 3066-3071.

Main, C. R.; Salvitti, L. R.; Whereat, E. B.; Coyne, K. J. Community-level and species-specific associations between phytoplankton and particle-associated *Vibrio* species in Delaware's inland bays. *Appl. Environ. Microbiol*. **2015**, *81(17*), 5703-5713.

Grimes, D. J.; Ford, T. E.; Colwell, R. R.; Baker-Austin, C.; Martinez-Urtaza, J.; Subramaniam, A.; Capone, D. G. Viewing marine bacteria, their activity and response to environmental drivers from orbit: satellite remote sensing of bacteria. *Microbial Ecology* **2014,** *67*(3), 489–500.

Hartwick, M., and Jones, S. H. *Vibrio parahaemolyticus* Population Dynamics Within an Estuarine Microbial Community. 2016. Poster presentation. International Symposium on Microbial Ecology, Montreal, Canada.

Hartwick, M.; Berenson, A.; Whistler, C.A.; Cooper, V.S.; and Jones, S.H. Seasonal Plankton Communities and Plankton-Associated *Vibrio parahaemolyticus* Improve the Estimation of *Vibrio parahaemolyticus* Concentration in Oysters in the Great Bay Estuary (in preparation)

Gilbert, J.A.; Steele, J.A.; Caporaso, J.G.; Steinbrück, L.; Reeder, J.; Temperton, B.; Huse, S.; McHardy, A.C.; Knight, R.; Joint, I.; Somerfield, P. Defining seasonal marine microbial community dynamics. *The ISME journal* **2012,** *6*(2), p.298.

Rehnstam-Holm, A.; Godhe, A.; Härnström, K..; Raghunath, P.; Saravanan, V.; Collin, B.; Karunasagar, I.; Karunasagar, I.(2010). Association between phytoplankton and *Vibrio* spp. along the southwest coast of India: a mesocosm experiment. *Aquatic Microbial Ecology* **2010**, 58(2), 127-139.

Davis, B. J., Jacobs, J. M., Zaitchik, B., DePaola, A., & Curriero, F. C. Vibrio parahaemolyticus in the Chesapeake Bay: operational in situ prediction and forecast models can benefit from inclusion of lagged water quality measurements. *Applied and environmental microbiology* **2019**, 85(17), e01007-19.

Schillaci C. 2016. *Vibrio parahaemolyticus* (Vp) Illness in Massachusetts and Impact on the Cape and Island's Shellfish Industry.4 TH ANNUAL CAPE COASTAL CONFERENCE, Waquoit Bay, MA. http://www.waquoitbayreserve.org/wp-content/uploads/CC4_Schillaci_Vibrio.pdf

Semenza, J.C.; Trinanes, J.; Lohr, W.; Sudre, B.; Löfdahl, M.; Martinez-Urtaza, J.; Nichols, G.L; Rocklöv, J. Environmental suitability of Vibrio infections in a warming climate: an early warning system. *Environmental health perspectives* **2017,** *125*(10), p.107004.

Vezzulli, L.; Grande, C.; Reid, P.C.; Hélaouët, P.; Edwards, M.; Höfle, M.G.; Brettar, I..; Colwell, R.R.; Pruzzo, C. Climate influence on Vibrio and associated human diseases during the past half-century in the coastal North Atlantic. *Proceedings of the National Academy of Sciences* **2016**, *113(34*), .E5062-E5071.

Vezzulli, L.; Pezzati, E.; Brettar, I.; Höfle, M.; Pruzzo, C. Effects of global warming on vibrio ecology. *Microbiology Spectrum* **2015**, 3(3): doi:10.1128/microbiolspec.VE-0004-2014

Oliver, T. H.; Roy, D. B. . The pitfalls of ecological forecasting. *Biological Journal of the Linnean Society* **2015,** *115*(3), 767-778

Table 6 (S1). Trend and seasonality estimates detected by Model 3 and Model 4 for *V. parahaemolyticus* concentrations and environmental variables (Model 3, top and Model 4, bottom).

Variable [a]	p-value	r^2	Deviance	AIC
Vp (MPN/g)	<0.001 0.002	0.24	0.27	673.4
	<0.001 <0.001 <0.001	0.48	0.49	582.65
Water Temperature (°C)	0.11 <0.001	0.56	0.59	772.1
	0.11 <0.001 <0.001	0.93	0.93	496.1
Dissolved Oxygen (mg/L)	0.82 <0.001	0.21	0.23	441.5
	0.87 <0.001 <0.001	0.58	0.59	352.0
Salinity (ppt)	<0.001 0.45	0.38	0.42	805.6
	<0.001 <0.001 0.01	0.48	0.51	781.2
pH	<0.001 0.16	0.18	0.23	10.1
	<0.001 0.54 0.41	0.18	0.24	12.9
Turbidity (NTU)	0.04 0.11	0.03	0.50	1713.6
	0.03 0.37 0.38	0.03	0.05	1715.9
Chlorophyll-*a* (μg/L)	<0.001 <0.001	0.24	0.30	758.3
	<0.001 0.67 <0.001	0.24	0.30	759.0
Total Dissolved Nitrogen (mg/L)	<0.001 0.01	0.46	0.49	-289.3
	<0.001 0.14 0.002	0.47	0.50	-289.9

	0.37 0.07	0.03	0.06	-76.4
Rainfall (mm)	0.29 0.24 0.02	0.04	0.08	-76.9

Table 7 (S2). Model variables, their significance and model fit evaluation for models based on combinations of photoperiod, day of study, harmonic regression and environmental variables. A. Gaussian. B. Negative binomial regression.

A. Model-Gaussian	Coefficient	r^2	Deviance	AIC
Model 1	0.57*** 0.0002***	0.19	0.20	666.4
Model 1 + sine + cosine	-4.1 0.0006*** -0.53 -1.6	0.51	0.52	596.9
Model 1 + Temp	-0.37** 0.0004*** 0.47***	0.57	0.57	577.6
Model 1 + Temp + Sal	-0.30** 0.0004** 0.44*** 0.06	0.57	0.58	577.0
Model 1 + Temp + C-pH	-0.3** 0.0003** 0.46*** -3.8***	0.61	0.61	564.4
Model 1 + Temp + C-pH + Sal	-0.3** 0.0002** 0.44*** -3.6** 0.02	0.61	0.62	565.9
Model 2+ Temp	0.0004*** 0.06 1.51 0.51***	0.56	0.57	579.7
Model 2 + Temp + Sal	0.0003** 0.23 1.5 0.5*** 0.06	0.57	0.58	578.9
Model 2 +Temp + C-pH	0.0003** 0.07 1.4 0.50*** -3.8***	0.61	0.61	566.3
Model 2+ Temp + C-pH + Sal	0.0003** 0.15 1.5 0.49*** -3.6** 0.03	0.60	0.62	567.7
B. Model-Negative binomial				
Model 1	0.6*** 0.0006***	0.19	0.17	1619.5
Model 1 + sine + cosine	-6.2** 0.0005*** 0.6 -0.3***	0.49	0.52	1532.8
Model 1 + Temp	-0.32** 0.0004*** 0.4***	0.56	0.53	1521.3
Model 1 + Temp + Sal	-0.27** 0.0004** 0.4*** 0.05*	0.56	0.53	1521.1
Model 1 + Temp + C-pH	-0.32*** 0.0003***; 0.4 -4.5	0.61	0.58	1501.9
Model 1 +Temp + C-pH + Sal	-0.32*** 0.0003*** 0.4*** -4.5*** -0.004	0.61	0.58	1503.9
Model 2 + Temp	0.0003*** -0.3 0.8 0.4***	0.55	0.52	1523.8
Model 2 + Temp + Sal	0.0003*** 0.03 1.1 0.4*** 0.05*	0.56	0.53	1523.5
Model 2 +Temp + C-pH	0.0003*** -0.3 0.8 0.4*** -4.5***	0.61	0.58	1504.2
Model 2 + Temp + C-pH + Sal	0.0003*** -0.3 0.8 0.4*** -4.6*** -0.006	0.61	0.58	1506.2

significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1

Chapter 2

The Seasonal Ecology of *Vibrio parahaemolyticus* and Plankton in a New Hampshire Estuary

Meghan Hartwick[a,b], *Audrey Berenson*[a,b], *Cheryl A. Whistler*[a,b], *Elena N. Naumova*[d], *Stephen H. Jones*[a,b,c]

[a] Northeast Center for *Vibrio* Disease and Ecology, University of New Hampshire, Durham, NH
[b] Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH
[c] Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH
[d] Division of Nutrition Data Sciences, Friedman School of Nutrition Science and Policy, Tufts University, Boston, MA; elena.naumova@tufts.edu

ABSTRACT

The emergence and establishment of *Vibrio parahaemolyticus* as a causative agent of shellfish-borne illnesses in the Northeast United States has stimulated keen interest in understanding the environmental conditions that may coincide with *V. parahaemolyticus*-associated-human health risk in this region. Seasonal water temperature is an important characteristic of *V. parahaemolyticus* dynamics and water temperature is used to manage shellfish harvesting-related health risks in this region. However, a wide range of environmental variables including copepods and chitinous phytoplankton in the water column, have also been reported as factors that affect *V. parahaemolyticus* dynamics, though little is known regarding their significance in the Northeast US. A holistic evaluation of the seasonal ecology of *V. parahaemolyticus* concentration was conducted using three years of plankton community analysis, data for nutrients, water quality and *V. parahaemolyticus* concentrations in water, sediment, and plankton from a site in the Great Bay estuary (GBE) to identify the environmental variables that may relate to *V. parahaemolyticus* dynamics in oysters. *V. parahaemolyticus* concentrations in sediment and phytoplankton, *Helicotheca tamensis, Rhizosolenia* spp., water temperature, salinity and PO_4 were identified as the optimal combination of variables to estimate the overall seasonality of *V. parahaemolyticus* concentration in oysters. Together, this provides the first comprehensive report of the microbial seasonal ecology of the GBE as it relates to plankton dynamics and *V. parahaemolyticus* concentrations in oysters. The results of this work can be applied to monitoring and mechanistic studies to improve our understanding of the seasonal dynamics that contribute to pre-harvest risk for *V. parahaemolyticus* disease in this region.

INTRODUCTION

The emergence and establishment of *Vibrio parahaemolyticus* as a causative agent of shellfish-borne illnesses in the Northeast United States has stimulated keen interest in understanding the environmental conditions that may pose a human health risk in this region. The ecology of *V. parahaemolyticus* has been the focus of numerous intensive studies and a wide array of

environmental variables associate with *V. parahaemolyticus* dynamics including water temperature, salinity, pH, inorganic and organic nutrients, suspended solids-turbidity, chlorophyll-*a*, light availability and meteorological conditions (Hartwick et al., 2019; Lovell, 2017; Urquhart et al., 2016; Turner et al., 2014; Martinez-Urtaza et al., 2012; Vezzulli et al., 2009, Jones and Summer-Brason, 1998, DePaola et al., 1990, Kaneko and Colwell, 1973). *V. parahaemolyticus* can be associated with shellfish, sediment, micro and macroplankton, crabs and protozoa. Though the majority of these associations are believed to be benign, abscesses and lesions attributed to *V. parahaemolyticus* disease are also documented in corals, dolphins, sea otters, finfish and shrimp (Erken et al., 2015, Vezzulli et al., 2013, Hughes et al., 2013; Martinez-Urtaza et al., 2008).

Sediments provide nutrients and protection from environmental stressors for *Vibrio parahaemolyticus*, especially during fall, winter and spring in cold and temperate water regions (Alipour et al., 2014; Vezzulli et al., 2009; Venkateswaran et al., 1990; Kaneko and Colwell, 1973). The water column is a common detection point for *V. parahaemolyticus*, though it is frequently the organically-enriched particulate fractions of the water column, including marine snow, detritus and suspended sediment, that are the point of interest for these studies (Williams et al., 2014; Froelich et al., 2013). This is based on findings that suggest that *V. parahaemolyticus* prefers an attachment versus free-living lifestyle and therefore more likely associates with these particles (Parveen et al., 2008; Vezzulli et al., 2009; Johnson et al., 2013).

Whether shellfish are a preferred environment for *Vibrio parahaemolyticus* remains unclear, however because of their commercial importance, the shellfish-*V. parahaemolyticus* relationship is the most studied interaction. (Lovell et al., 2017; DePaola et al. 1990, 2003; Zimmerman et al. 2007). Filter feeding of suspended material is the most likely route by which *V. parahaemolyticus* becomes concentrated in shellfish (e.g., Froelich et al. 2013). Kaneko and Colwell (1977) first described how this filter feeding likely contributes to the accumulation of *V. parahaemolyticus* in the Chesapeake Bay as a seasonal cycle between sediment, water, plankton and shellfish. Given this, copepods and chitinous phytoplankton or water quality variables, such as organic and inorganic nutrients and chlorophyll-*a* that have often been associated with phytoplankton abundance (Caburlotto et

al., 2010; Takemura et al., 2014) are frequently studied as they may relate to *V. parahaemolyticus* dynamics (Turner, 2009 and 2014, Paranjpye et al., 2015 and Gilbert et al., 2012; Martinez-Urtaza et al., 2012, Asplunde et al. 2011, Beveridge et al., 2010, Kaneko and Colwell, 1975).

As *Vibrio parahaemolyticus* disease dynamics are changing worldwide, plankton dynamics, including the distribution of plankton taxa, and the frequency and intensity of harmful algal blooms are also changing, concurrent with climate related changes in the marine environment (Cavicchioli et al., 2019; Townhill et al., 2018; Baker-Austin et al., 2017; Grimes et al., 2014; Vezzulli et al., 2013). In the GBE, *V. parahaemolyticus* concentrations are increasingly elevated throughout the summer and are remaining high late into fall months (Hartwick et al., 2019; Urquhart et al., 2016). Given the complex ecology of *V. parahaemolyticus* and well-documented association with plankton, an extensive study of the abiotic and biotic ecology of *V. parahaemolyticus*, including the plankton community and nutrients, of the ecology of *V. parahaemolyticus* in the Great Bay Estuary (GBE) (Figure 9) was undertaken beginning in 2014.

Figure 9. The GBE Study area on the border of Maine and New Hampshire. OR = Oyster River; NI = Nannie Island

Environmental variables were assessed individually and together to determine how each variable contributes to the progression of conditions over the course of a season that may lead to the

accumulation of *Vibrio parahaemolyticus* in oysters and characterize the environmental factors that may be driving the changing dynamics of *V. parahaemolyticus* in the GBE. This study represents the first comprehensive, multi-year assessment of the seasonal microbial ecology of *V. parahaemolyticus* concentrations in the GBE. The outcomes of this work contribute to a better understanding of the conditions that may contribute to *V. parahaemolyticus* dynamics in the Northeast, US and identifies new targets for monitoring and mechanistic studies to understand human health risks.

METHODS

Study sites, environmental sampling and bacterial analysis

The study area was the Great Bay estuary of New Hampshire, focused at a site near Nannie Island (NI) that has a significant oyster (*Crassostrea virginica*) bed and is a long-term monitoring location (Urquhart et al. 2016; Jones and Summer-Brason 1998). The estuary has been monitored for over 30 consecutive years (March-December) by multiple agencies, including the Great Bay National Estuarine Research Reserve (GBNERR) and their System Wide Monitoring Program (SWMP). Continuous (Q15) water temperature, salinity, dissolved oxygen (DO), pH, and turbidity data were obtained from SWMP from 2014-2016 for times simultaneous with and preceding sampling events. Nutrient and related parameter data for grab samples; Non-Purgeable Organic Carbon (NPOC), Total Dissolved Nitrogen (TDN), Nitrate and Nitrite (NO_3 + NO_2,) Ammonium (NH_4), Orthophosphate (PO_4), Dissolved Organic Nitrogen (DON), Total Suspended Solids (TSS), Particulate Carbon (PC), Particulate Nitrogen (PN), chlorophyll-*a* (CHL) and pheophytin (PHEO) measurements were obtained from the SWMP database (https://cdmo.baruch.sc.edu/dges/).

Oyster, sediment, water and plankton samples, referred to as matrices for *Vibrio parahaemolyticus,* were collected concurrently with time of sample water temperature, salinity, pH and dissolved oxygen (DO) measurements using YSI 6600 and EXO multiprobe sondes (Yellow Springs Instruments, Yellow Springs, Ohio), during low tide from the NI study site. Plankton samples were collected using a 53 μm mesh net (Aquatic Instruments). Sediment, oyster, phyto- and zooplankton samples were processed and analyzed for *Vibrio* detection according to previously

published methods with alkaline peptone water enrichment, Vibrio CHROMAgar and the FDA established PCR (Hartwick et al. 2019) and MPN calculations at Jackson Estuarine Lab (JEL). Probable *Vibrio* isolates were confirmed as *V. parahaemolyticus* by PCR detection of the *tlh* gene (Urquhart et al., 2016, Hartwick et al., 2019, Kaysner and DePaola (BAM), 2004).

Plankton collection and phototactic separation

Plankton samples were collected in duplicate using a 53 μm mesh net and three different methods for collecting adequate and increasingly more accurate volumes of water: in 2014 a Student Net (Aquatic Instruments, Florida USA), was used during ten weighted tows to collect ~140L of water; for March 2015 through Oct 2015 a Niskin sampler with Student Net (Aquatic Instruments, Florida USA) was used to collect 160L; and for October 2015 through December 2016 a 30L Schindler Patalas (Wildco, Florida, USA) was used to collect 180L. One sub-sample was phototactically separated to provide efficient separation of the phytoplankton from the zooplankton fractions using a method and device for plankton separation (Leland and Haney, 2018). Time series trials were performed in triplicate at 0, 10, 20, 30, 40 minutes with optimum separation efficiency being observed at 40 minutes. This separation time was in agreement with previous observations (Nancy Leland pers. comm). Sample one, separated into phyto- and zooplankton fractions was filtered in 53 um pore size hand-prepared filter cones using Nitex Bolting Cloth (Wildco, Florida, USA), concentrated and weighed. Zoo and phytoplankton samples were processed identically to oyster, water and sediment samples accordingly to previously published methods (Urquhart et al., 2016, Hartwick et al., 2019, Kaysner and DePaola, 2004) to determine *Vibrio parahaemolyticus* MPN/g. The subsample was then resuspended to a volume of 50 mL in <53 μm filtrate water from NI and preserved with 1% sucrose formalin (US EPA, 2003) for sample community identification, enumeration and biomass concentration (cells/L) determination.

Plankton biomass and community analysis

Plankton community enumeration and concentration determinations were performed by methods consistent with standard methods for plankton analysis (USEPA, 2003) using a phase-contrast microscope and a 1mL grafted Sedgwick rafter (Wildco, Florida USA). Plankton

identification was confirmed with a combination of identification key resources including: Dolan and Cooper, 2006 and Baker et al., 2012. Samples were analyzed with a phase-contrast microscope at 400x magnification (Olympus, USA) to count 10 non-consecutive columns (100 cells total). Sample dilution was conducted when necessary, for samples too abundant for accurate counting using deionized (DI) water. Counts were transformed to cells/L for each taxa observed in the sample

Statistical analysis

All statistical computations were performed in the R Statistical Program and Environment, version 3.5.3 (R Core Team, 2019) and vegan: Community Ecology Package, 2.5-2 (Oksanen et al., 2018). Multiple steps of data analysis were performed to evaluate the relationship between plankton, environmental determinants and *V. parahaemolyticus* concentrations in the GBE. Significance for all analysis was determined by $p < 0.05$.

Environmental variables

MPN values for *Vibrio parahaemolyticus* concentrations were log-transformed for analysis to approximate normality and reduce skewness. Total phytoplankton and zooplankton/L were determined by subtracting the total number of nauplii, copepods and *Tintinnida* from the total plankton abundance/L. The *V. parahaemolyticus* concentration associated with a gram wet weight of phyto or zooplankton was transformed to *V. parahaemolyticus* concentration associated with phyto or zooplankton/L by dividing the MPN by the number of L required to produce one gram of phyto or zooplankton. Relative concentrations between matrices were compared by two-way analysis of variance (ANOVA).

Plankton sample and community analysis

Total Abundance (TA) and Relative Abundance (RA), species richness, evenness and Shannon's H were calculated for the entire study period. Taxa observed in collected samples were assessed as Abundant (> 4% of total), Common (≤ 4% and ≥ 0.1%) or Rare (<0.1%) in the GBE plankton community according to the DAFOR semi-quantitative abundance classification. Plankton counts were log + 1 transformed to reduce skew and kurtosis. Rare taxa (abundance <0.1%) were not

included in community analysis or structural equation modeling. Seasonal community assemblage was assessed between calendar seasons (spring: March to June 21st, summer: June 22nd through September 21st and fall: September 22nd to December, and years (2014, 2015 and 2016) by Permanova, Multi Response Permutation Procedure (MRPP), and Indicator Species Analysis (ISA).

Seasonality

The seasonality and general trend in the environmental variables were explored with:

$$\text{Model 1: } E(Y_t) = \beta_0 + \beta_1 t + \beta_p Photoperiod, \text{ and} \quad (1)$$

$$\text{Model 2: } E(Y_t) = \beta_0 + \beta_1 t + \beta_s \sin(2\pi\omega t) + \beta_c \cos(2\pi\omega t). \quad (2)$$

to fit periodicity and trends of the seasonal oscillations (Hartwick et al., 2019). In both models, Y_t is the daily time series for the outcome of interest, β_0 is the intercept, t is the daily time series, $\beta_1$indicated a general trend in the variable of interest. Model 1 contains the photoperiod variable $\beta_p Photoperiod$ and Model 2 uses harmonic regression terms for the calendar day in the study where βs and βc are the coefficients of the harmonic terms and ω is the term representing the annual cycle (365.25 days, ω=1/365.25). The peak timing of the periodic oscillations identified by Model 2 was determined by calculating the phase shift:

$$\psi = \arctan\left(\frac{\hat{\beta}s}{\hat{\beta}c}\right) + k. \quad (3)$$

When $\hat{\beta}_S$ and $\hat{\beta}_c$ were positive, k = 0. When $\hat{\beta}_S < 0$ and $\hat{\beta}_c > 0$, $k = 2\pi$ and when $\hat{\beta}_S$ and $\hat{\beta}_c$ were negative, or when $\hat{\beta}_S > 0$ and $\hat{\beta}_c < 0$, then $k = \pi$. The phase shift (ψ) was then multiplied by 365.25. Confidence intervals (CI) of the peak timing were calculated by determining the estimated variance of the phase shift (ψ) where ($\sigma_{\beta_c\beta_s}$) is the covariance and $\hat{\beta}_s^2$ and $\hat{\beta}_c^2$ are the variances of β_s and β_c in the following equation:

$$var(\psi) = \frac{(\sigma_{\beta s}\beta c)^2 + (\sigma_{\beta c}\beta s)^2 - (2\sigma_{\beta_c\beta_s}\beta_s\beta_c)}{(\hat{\beta}_s^2 + \hat{\beta}_c^2)(\hat{\beta}_s^2 + \hat{\beta}_c^2)^2}. \quad (4)$$

CI values for the peak timing estimates were determined as: $1.96 * \sqrt{Var(\psi) * 365.25/2\pi}$. The seasonality of the environmental variables was evaluated by the significance of the coefficients,

the deviance explained, AIC, and coefficient of determination (r^2) value of Model 1 and 2 (Naumova et al., 2007, Alarcon Falconi et al., 2018).

Correlation

The Mantel test and Spearman rank correlation analysis were performed for *Vibrio parahaemolyticus* concentrations in all matrices, water quality, dissolved nutrients and plankton community data to assess the relationship of environmental variables to *V. parahaemolyticus* dynamics in oysters.

Systems ecology modeling

Partial Least Squared Residual models (PLSR) were used to identify the most significant seasonal variables associated with *Vibrio parahaemolyticus* concentration in oysters based on a variable importance score (VIP) >0.8. PLSR model performance was evaluated by Residual Mean Square Error (RMSE) and deviance explained. The variables identified by PLSR were integrated into structural equation models (SEM) to evaluated and explore potential drivers of the seasonal accumulation of *V. parahaemolyticus* in oysters in the GBE. A correlation table was provided to illustrate the correlation matrix of the environmental variables of interest. SEM model fit was evaluated based on the relationships between environmental variables as they were hypothesized by the SEM model and then compared to the actual environmental variable relationships that were observed in the correlation matrix. Chi-square ($\chi 2$) > 0.05 indicates that there is no significant difference between the observed correlation matrix and specified variable relationships in the SEM and therefore is a good representation of the relationships of the environmental variables. SEM model fit where $\chi 2$ $p > 0.05$ were then evaluated by model likelihood (H_1 compared to H_0), Comparative Fit (>= 0.90) Tucker Lewis Fit (>= 0.95), Standardized Root Mean Square Residual (SRMR < 0.08) AIC, and the coefficient of determination (r^2).

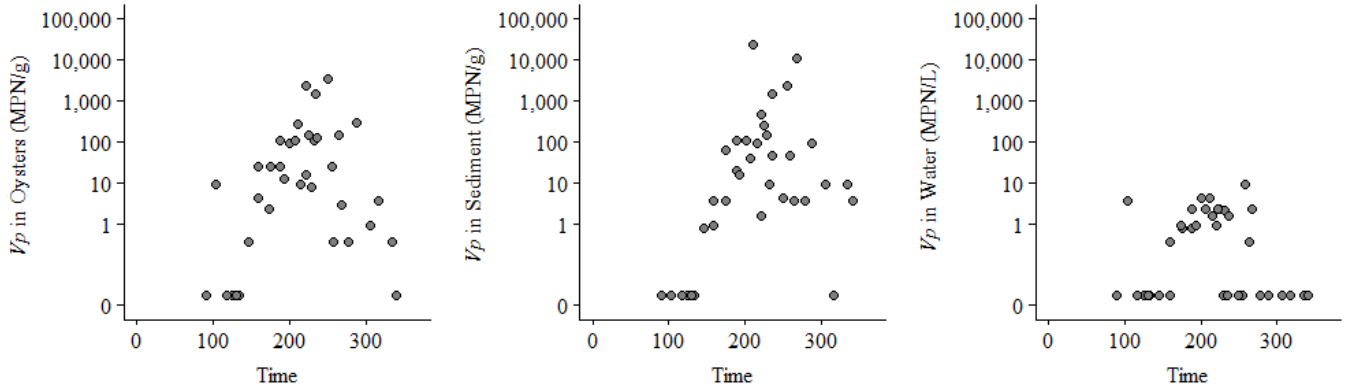
RESULTS

Vibrio parahaemolyticus, total plankton and environmental variable detection and timing

Water and other ecosystem matrices were analyzed for water quality conditions, plankton community abundance and *Vibrio parahaemolyticus* concentrations to understand the seasonal

microbial ecology of the GBE. Yearly sampling from 2014 through 2016 began when small-craft vessels could be safely operated dependent on ice out and seasonal conditions beginning in March (2016), May (2015) or July (2014) and ended in November (2015) or December (2014 and 2016). There were thirty-one total sampling events with complete data for *V. parahaemolyticus* concentrations, plankton community analysis, and water quality and nutrient data.

In July 2014 when this study began, *Vibrio parahaemolyticus* was detected in all matrices. Initial detection in 2015 was in early June from sediment, oysters and plankton, whereas *V. parahaemolyticus* was first detected in oysters and water in April 2016. Overall, *V. parahaemolyticus* was detected in 87.1% of samples from oysters (n=27), 83.3% of sediment samples (n= 26), 54.8% of phytoplankton samples (n =17), 45.1% of zooplankton samples (n = 14) and 45.1% of water samples (n=14), but not necessarily on the same dates. The timing of detection of *V. parahaemolyticus* in samples collected early in each year was variable between matrices. Total plankton abundance per sample ranged from 83 to 35,853 cells/L and the detection of *V. parahaemolyticus* from zoo or phytoplankton did not necessarily coincide with plankton abundance in the water column (Figure 10).



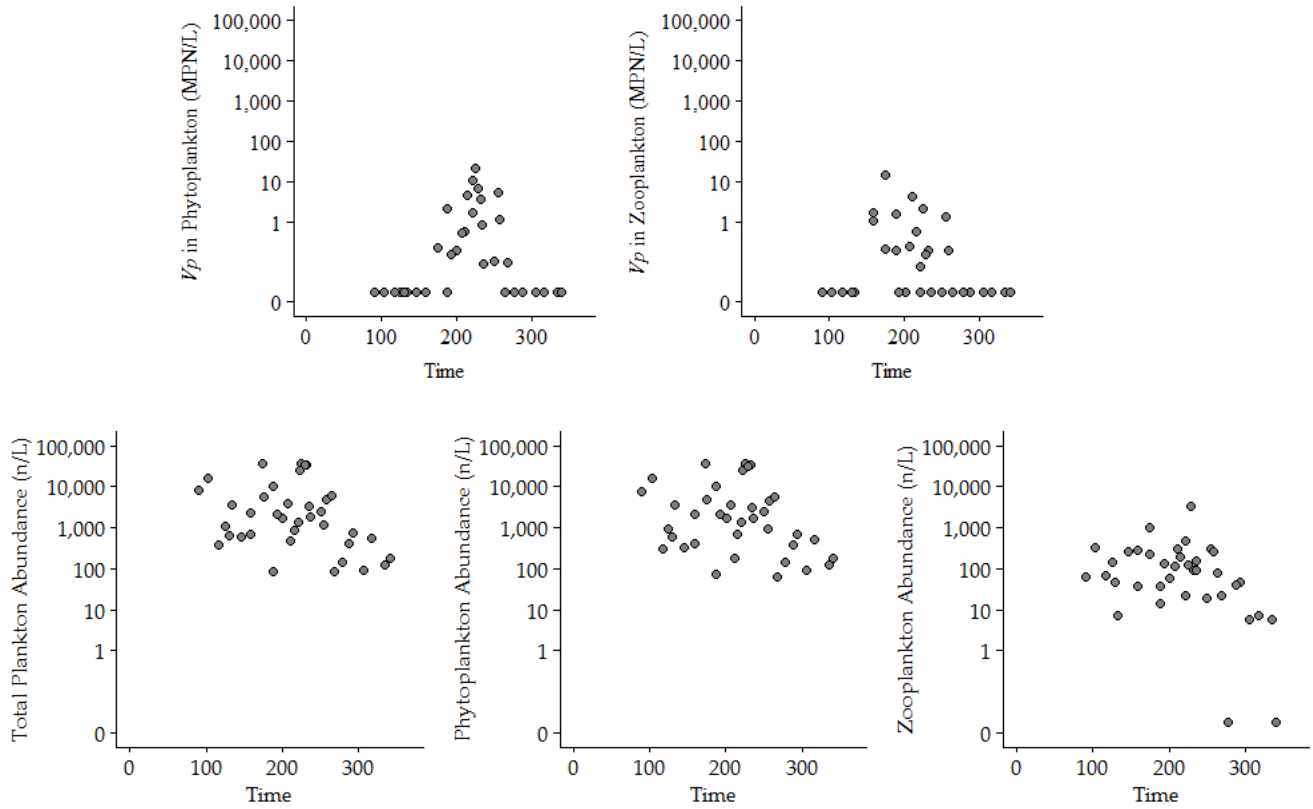


Fig. 10. *V. parahaemolyticus* concentration and plankton abundance superimposed by year. *V. parahaemolyticus* was cultured from (a) oyster, (b) sediment, (c) water, (d) phyto- (e) zooplankton and plankton was collected from the water column (f) total plankton, (g) phytoplankton and (h)zooplankton at Nannie Island between 2014 -2016.

The combined concentrations of *Vibrio parahaemolyticus* associated with phyto- and zooplankton were similar to the *V. parahaemolyticus* concentrations measured in the water from the same sample. The relative *V. parahaemolyticus* concentration (per g material versus per L water) was higher in oysters and sediment than in water, phyto and zooplankton ($p < 0.001$). Detection frequency and total abundance was higher for phytoplankton compared to zooplankton (Table 8).

Table 8. Range and mean values for *V. parahaemolyticus*, water quality and nutrients.

Variable	Min	Max	Mean and SD
Vp and Plankton			
Oyster Vp MPN/g	0.018	3500	249±722
Sediment Vp MPN/g	0.018	24000	1123±4342
Water Vp MPN/L	0.018	9.3	1.2±1.8
Phytoplankton Vp MPN/L	0.018	14	1.7 ± 4
Zooplankton Vp MPN/L	0.018	21	8.5±2.6

Total Plankton Abundance n/L	83	35853	6700 ±11229
Phytoplankton Abundance n/L	62	35630	6474±10980
Zooplankton Abundance n/L	0	3350	227±10980
Nutrient			
DON (mg/L)	0.0	0.2	0.12 ± 0.05
NH_4 (mgN/L)	0.0	0.1	0.02 ± 0.02
$NO_3 + NO_2$ (mgN/L)	0.01	0.2	0.04 ± 0.04
NPOC (mg/L)	0.36	3.8	2.31 ± 0.7
PC (mg/L)	0.43	3.6	1.16 ± 0.7
PN (mg/L)	0.05	0.5	0.17 ± 0.1
PO_4 (mgP/L)	0.00	0.07	0.03 ± 0.02
TDN (mg/L)	0.06	0.34	0.18 ± 0.06
Water Quality			
Chlorophyll-a (μg/L)	1.3	22.6	6.3 ± 4.5
Dissolved Oxygen (mg/L)	6.5	11.5	8.5 ± 1.3
pH	7.5	8.0	7.8 ± 0.15
Pheophytin (μg/L)	0.7	9.8	2.9 ± 2.2
Salinity (ppt)	14.1	32.1	27.1 ± 3.5
Water Temperature (°C)	6.3	25.3	17.7± 5.1
Total Suspended Solids	10.7	76.4	29.8 ± 15.6
Turbidity (NTU)	1.15	163.4	10.8 ± 13.3

Water temperatures ranged from 6.3°C to 25.3°C for sample dates during July 2014 to December 2016 of the study period (Table 1). The average salinity was 27.1±3.5 and ranged from 14.1 to 32.1 ppt, with the highest salinities occurring in mid to late September. pH values ranged between 7.5 and 8.0 and the dissolved oxygen concentration ranged from 6.5 to 11.5 mg/L. Chlorophyll-*a* and nutrient concentrations were generally consistent with data in previous reports (Hartwick et al., 2016; Urquhart et al., 2016; PREP, 2018). The average TDN concentration was 0.18± 0.06 mg/L and the average PO_4 concentration was 0.03±0.02 mg/L. Chlorophyll-*a* concentrations were generally low, with an average of 6.3±4.5 μg/L although one sample collected in September 2015 had a relatively high concentration of 22.2 μg/L.

Overall and seasonal plankton community dynamics

Thirty-four taxa of diatoms, dinoflagelletes and zooplankton comprised 76.5%, 11.8% and 11.8% of the relative abundance, respectively. The majority (77.8%) of phyto- and zooplankton taxa observed during this three-year period had been previously identified in the GBE (Jones, 2000;

NAI, 1979) (Table 9). *Cheatocerus spp.*, *Heliocotheca tamensis*, *Navicula spp.* and *Skeletonema spp.* had the highest relative abundance (RA), comprising 89.8% of the total abundance observed in the analyzed samples. *Fragilariopsis spp.* (RA = 1.7%) and *Navicula spp.* (RA = 14.5%) were present in all samples. Fifteen plankton taxa including: *Gymnodium spp*. *Prorocentrum* and *Eucampia,* were present in fewer than 0.1% of samples.

Table 9. Phytoplankton and zooplankton taxa, type, abundance and frequency in the GBE.

Organism	Type	Observed Historically	Annual	Classification	Total Abundance (total cells)	Relative Abundance (%)	Frequency
Chaetoceros spp.	Diatom	Yes	Yes	Abundant	145262	58.6	0.81
Navicula spp.	Diatom	Yes	Yes	Abundant	36102	14.6	1.00
Helicotheca tamensis	Diatom	No	Yes	Abundant	22098	8.9	0.89
Skeletonema spp.	Diatom	Yes	Yes	Abundant	19192	7.7	0.53
Tintinnida	Zooplankton	Yes	Yes	Common	4946	2.0	0.65
Fragilariopsis spp.	Diatom	Yes	Yes	Common	4385	1.8	1.00
Nauplii	Zooplankton	Yes	Yes	Common	2662	1.1	0.89
Coscinodiscus spp.	Diatom	Yes	Yes	Common	2396	1.0	0.86
Pleurosigma spp.	Diatom	No	Yes	Common	2020	0.8	0.78
Thalassiosira spp.	Diatom	Yes	Yes	Common	1596	0.6	0.20
Thalassionema spp.	Diatom	Yes	Yes	Common	1347	0.5	0.27
Cylindrotheca spp.	Diatom	Yes	Yes	Common	896	0.4	0.51
Licmophora spp.	Diatom	Yes	Yes	Common	846	0.3	0.54
Rhizosolenia spp.	Diatom	Yes	Yes	Common	833	0.3	0.57
Copepods	Zooplankton	Yes	Yes	Common	792	0.3	0.78
Stephanopyxis spp.	Diatom	No	Yes	Common	531	0.2	0.35
Bacillaria spp.	Diatom	Yes	Yes	Common	450	0.2	0.48
Biddulphia spp.	Diatom	Yes	Yes	Common	403	0.2	0.48
Ditylum spp.	Diatom	Yes	No	Common	274	0.1	0.24
Grammatophora spp.	Diatom	Yes	Yes	Rare	141	0.06	0.19
Leptocylindrus danicus	Diatom	No	Yes	Rare	112	0.05	0.02
Odontella spp.	Diatom	No	No	Rare	100	0.04	0.13
Ceratium spp.	Dinoflagellette	Yes	Yes	Rare	72	0.03	0.11
Detonula spp.	Diatom	Yes	No	Rare	51	0.02	0.19
Phaeocystis	Diatom	Yes	No	Rare	50	0.02	0.11
Pseudo-nitzschia spp.	Diatom	Yes	No	Rare	48	0.02	0.05
Dinobryon spp.	Diatom	Yes	No	Rare	28	0.01	0.11
Eucampia spp.	Diatom	Yes	No	Rare	17	0.01	0.03
Asterionellopsis spp.	Diatom	Yes	No	Rare	14	0.01	0.11
Prorocentrum spp.	Dinoflagellette	Yes	No	Rare	13	0.01	0.03
Gonyaulax spp.	Dinoflagellette	No	No	Rare	11	<0.01	0.03

Corethron spp.	Diatom	Yes	No	Rare	3	<0.01	0.03
Gymnodinium spp.	Dinoflagellette	Yes	No	Rare	3	<0.01	0.3
Cladocerans	Zooplankton	Yes	Yes	Rare	3	<0.01	0.01

Plankton abundance and detection were seasonally variable. On average, 13.8 taxa were detected (min= 6, max = 21) in each sample. Spring and fall plankton communities were generally more diverse (Shannon's H > 2) with lower total abundance, and overall, Shannon's H was inversely related to total abundance (R= -0.67). The concentration of *Chaetoceros* spp. and *Heliocotheca tamensis* was higher in summer than in spring or fall, whereas spring samples had higher concentrations of *Skeletonema* spp., *Biddulphia* spp., *and Stephanopyxis* spp. (Fig. 11). Plankton samples with the highest abundance, lowest diversity (Shannon's H <2) and lowest species richness were collected either during summer when *Heliocotheca tamensis* and *Cheatocerus* spp., were dominant or during spring when up to 97.3% of identified taxa were *Skeletenoma* spp. (abundance >7,700 cells/L) (Figure 3). Community assemblages and taxa-specific blooms re-occurred in 2014, 2015 and 2016.

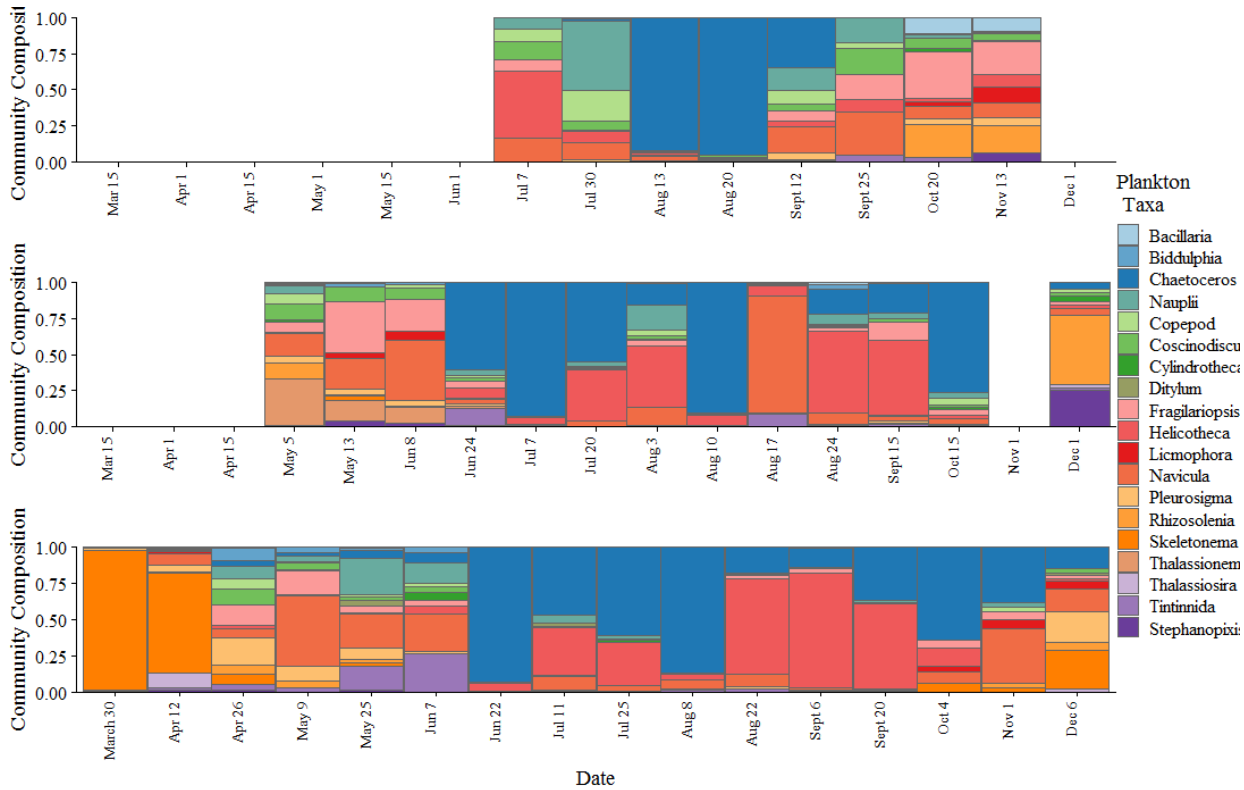


Figure. 11. Seasonal patterns of plankton abundance and composition at Nannie Island in (a) 2014, (b) 2015 and (c) 2016.

Multivariate assessment identified plankton whose abundance differed by season (MRPP, p <0.001) and year (MRPP, p= 0.008). Season specific differences in the plankton community were shown by ISA. *Chaetoceros* spp. (p = 0.01) and *Helicotheca tamensis* (p = 0.005) indicated the summer plankton community. The spring plankton community was indicated by the abundance of *Skeletonema spp*. (p = 0.015), *Biddulphia* (p = 0.005) and *Stephanopixis* (p = 0.01). *Rhizosolenia* (p = 0.01) indicated the fall plankton community. *Bacillaria, Cylindrotheca, Detonula, Ditylum, Fragiliaropsis, Licmophora, Thalassiosira* and *Tintinnida* were frequently detected in plankton samples but there was no season-specific difference in abundance. *Cosconodiscus* and *Thalassionema* abundance were significantly different between years (p = 0.008) though the abundance of these species had no influence on the season-specific plankton community defined by ISA (Table 10).

Table 10. Multivariate analysis of the plankton community composition compared between seasons and years.

Group	Pairwise Permanova p(adj)	Indicator Species
Season		
Summer vs Fall and Spring	0.003	*Chaetoceros*** *Helicotheca***
Summer vs Spring	0.003	*Skeletonema*** *Biddulphia*** *Stephanopyxis***
Fall vs Spring	0.006	*Rhizosolenia***
Year		
2014 vs 2015	0.882	0
2014 vs 2016	0.039	*Coscinodiscus***
2015 vs 2016	0.048	*Coscinodiscus*** *Thalassionema***

[a] significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1

Seasonality of environmental variables in the GBE

Seasonality was assessed by using a photoperiod (Model 1) and a harmonic regression model (Model 2) for all environmental variables to determine whether variables displayed consistent

annual periodicity. In general, environmental variables with well-pronounced seasonality were well fit by either Model 1 or Model 2 and had well described seasonal peak timing based on the relative estimation and breadth of confidence intervals. Some environmental variables were well described by both models, and peak timing was typically estimated to occur on day 170, thus nearly coincident with the peak timing of the photoperiod variable (Table 11 and S1). 40% of the variance observed in *Vibrio parahaemolyticus* in zooplankton was explained by Model or Model 2, whereas 6% and 60% of the variance observed in *V. parahaemolyticus* in oysters was explained by Model 1 and Model 2, respectively. Since the peak timing of *V. parahaemolyticus* in zooplankton occurs on day 188 ± 34 and the peak timing of *V. parahaemolyticus* in oysters occurs on day 230 ± 13 it is probably that a lag between the peak timing of photoperiod (Day 170) and the peak timing of *V. parahaemolyticus* in oysters must be accounted for to provide the same performance observed for the estimation of *V. parahaemolyticus* in zooplankton by Model 1.

Table. 11. Variables that demonstrate significant seasonality based on photoperiod (Model 1, first row) and harmonic regression (Model 2, second row) modeling.

Variable[a]	Coefficient[b]		Standard Error		r^2	Deviance Explained	AIC	Peak timing[c]
	trend	seasonality	trend	seasonality				
Vp Oyster	0.001	0.41	0.002	0.34	0.04	0.06	198.65	
	0.001	-3.80*** -3.61***	0.002	0.64 0.79	0.55	0.59	161.06	230 ± 13
Vp Sediment	-0.005	-0.005	0.002	0.35	0.07	0.12	202.12	
	-0.003*	-4.42*** -2.60***	0.002	0.63 0.77	0.63	0.66	169.7	243 ± 15
Vp Phyto	-0.003	0.29	0.001	0.21	0.20	0.24	156.89	
	-0.002**	-1.89*** -2.16***	0.001	0.43 0.54	0.53	0.58	138.7	224 ± 16
Vp Water	-0.002	0.49**	0.001	0.21	0.17	0.22	161.99	
	-0.001	-1.05* -2.27***	0.001	0.53 0.65	0.29	0.35	157.3	207 ± 22
Vp Zoo	-0.003**	0.45**	0.001	0.16	0.36	0.40	130.86	
	-0.002**	-0.14*** -1.55***	0.001	0.47 0.55	0.35	0.41	132.36	188 ± 34
Total Plankton	<0.001	0.43	<0.001	0.15	0.15	0.20	147.29	
Abundance	<0.001	-0.09 -1.54**	0.001	0.44 0.53	0.15	0.22	148.33	186 ± 32
Phytoplankton	<0.001	0.41*	0.001	0.16	0.11	0.16	151.87	
Abundance	<0.001	-0.12 -1.48**	0.001	0.47 0.57	0.11	0.18	152.98	187 ± 35
Zooplankton	<0.001	0.53***	0.001	0.13	0.32	0.35	135.59	
Abundance	<0.001	0.01 -1.79***	0.001	0.37 0.45	0.32	0.37	136.67	182 ± 24
Helicotheca tamensis	<0.001	<0.001	<0.001	<0.001	0.05	0.10	176.70	
	0.002*	-2.7*** -3.3***	0.001	0.5 0.5	0.59	0.63	150.1	223 ± 11
Rhizosolenia spp.	-0.002	-0.33**	0.001	0.16	0.11	0.16	141.93	
	-0.003***	1.5*** 2.1***	0.001	0.3 0.4	0.51	0.55	127.2	221 ± 14
Chaetoceros spp.	0.002	0.53	0.002	0.31	0.04	0.09	191.63	
	0.004**	-2.4*** -3.4***	0.002	0.7 0.9	0.34	0.39	185.1	217 ± 18
Copepod	-0.002*	0.22	<0.001	0.12	0.2	0.28	124.84	

	-0.001*	-0.32 -0.92*	<0.001	0.34 0.42	0.25	0.31	124.96	202 ± 36
Nauplii	-0.001	0.51***	<0.001	0.14	0.27	0.32	136.92	
	-0.001	-0.5** -1.9***	0.001	0.3 0.5	0.34	0.39	137.1	196 ± 20
Ditylum	0.002**	0.24	<0.001	0.12	0.18	0.23	122.02	
	0.002**	-0.11 0.88*	<0.001	0.33 0.41	0.18	0.25	123.26	190 ± 40
Navicula	-0.002	0.39***	<0.001	0.14	0.15	0.20	134.43	
	-0.002	0.20 -1.21*	<0.001	0.39 0.48	0.13	0.21	136.09	173 ± 39
Biddulphia	<0.001	0.28*	<0.001	<0.001	0.14	0.09	137.79	
	-0.001	1.12** -0.27	<0.001	0.38 0.46	0.19	0.26	132.66	105 ± 48
Coscinodiscus	-0.003***	0.24	<0.001	0.13	0.4	0.44	128.20	
	-0.004***	1.00** -0.24	<0.001	0.33 0.41	0.5	0.55	122.62	105 ± 47
Thalassionema	-0.002	0.27	0.001	0.18	0.08	0.13	152.77	
	-0.002*	1.18** -0.19	0.001	0.48 0.59	0.17	0.24	149.66	101 ± 58
Pleurosigma	0.003	0.19	0.001	0.18	0.02	0.04	150.79	
	-0.0003	1.37** 0.14	0.001	0.44 0.54	0.17	0.24	144.21	85 ± 43
Skeletonema spp.	0.003	0.03	0.001	0.22	0.03	0.09	167.11	
	0.002	1.8*** 0.9***	0.001	0.5 0.3	0.29	0.35	160.8	64 ± 31
Stephanopixis	-0.002	-0.08	0.001	0.16	0.01	0.07	143.75	
	-0.03	2.7** 1.5	0.14	0.84 1.04	0.20	0.27	156.43	62 ± 32
PO_4	<0.001	<0.001	<0.001	<0.001	0.05	0.03	161.06	
	<0.001	-0.007*** -0.008*	<0.001	0.003 0.003	0.63	0.66	199.33	253 ± 16
Salinity	0.004	-0.33	0.002	0.37	0.05	0.11	179.22	
	0.005**	-4.6*** -2.2***	0.002	0.5 0.7	0.76	0.78	134.87	248 ± 2
Dissolved Oxygen	<0.001	-0.18	<0.001	0.13	0.02	0.08	112.91	
	<0.001	1.7*** 1.8***	<0.001	0.03 0.04	0.83	0.84	56.42	227 ± 7
Water	<0.001	1.65	0.002	0.47	0.27	0.32	195.15	
Temperature	<0.001	-5.8*** -9.7***	0.0002	0.37 0.49	0.94	0.95	115.86	213 ± 2
$NO_3 + NO_2$	<0.001	0.01***	<0.001	<0.001	0.37	0.41	119.35	
	<0.001	0.05* 0.07***	0.008	0.009 0.04	0.47	0.52	126.39	200 ± 14

[a] Variable are shown for Model 1, first row and Model 2, second row for sine and cosine terms; [b] significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1; [c] Peak timing (day of year) estimates are represented by the mean and standard error values; for two parameters DO and TDN the estimates reflect the seasonal nadir.

Vibrio parahaemolyticus concentrations were markedly seasonal in all matrices, though with varying strength and peak timing. Model 2 fit *V. parahaemolyticus* dynamics in oysters, sediment, phytoplankton and zooplankton better than Model 1, with the harmonic regression explaining > 58.0% of the deviance. The peak timing for *V. parahaemolyticus* concentrations in oysters was estimated at day 230 ± 13 days, whereas the peak timing of *V. parahaemolyticus* concentrations associated with phytoplankton occurred approximately one week prior at 224 ± 16 and *V. parahaemolyticus* concentrations in sediment two weeks after at 243 ± 15. The seasonality of *V. parahaemolyticus* concentrations in water and zooplankton was fit by both Models 1 and 2, though

the strength of seasonality was less pronounced and the peak timing in these matrices occurred at least three weeks prior to other matrices (days 207 ± 22 and 188 ± 34, respectively). The seasonality of total plankton, phytoplankton and zooplankton abundances also varied, though the peak timing estimates were similar (ranging from day 182 to 187). Models 1 and 2 both fit the seasonality of zooplankton abundance well, while seasonality was less pronounced for total plankton and phytoplankton abundance. Deviance explained by either model was >35% for zooplankton abundance compared to < 22% deviance explained by seasonality for phytoplankton or total plankton.

Considered individually, the seasonal variability of eleven of the sixteen abundant or moderately abundant phytoplankton taxa was well fit by Model 1 or Model 2. As expected, peak timing of the abundance of individual plankton taxa was widely distributed throughout the course of a year. The seasonality of the majority of phytoplankton taxa was best fit by Model 2. For example, 63% of the deviance in *Helicotheca tamensis* was explained by Model 2 and the peak timing was estimated at 223 ± 11, whereas only 10% was explained by the photoperiod model (Model 1). Similarly, *Chaetoceros* spp. variability was well fit by the Model 2 compared to Model 1 (39% and 9%, respectively) and had similar seasonal peak timing to *Helicotheca tamensis. Skeletonema* spp. was also well described by Model 2 (variability explained: 35% compared to 9%), though estimated peak timing was earlier in the year (day 64 ± 31) compared to the majority of other phytoplankton taxa. Models 1 and 2 both provided good fit for *Navicula* spp. seasonality and nauplii, though peak timing for *Navicula* spp. was approximately twenty days earlier at day 173 ± 39 compared to the peak timing for nauplii (day 196 ± 20). The seasonality of *Bacillaria* spp., *Cylindrotheca* spp., *Licmophora* spp., *Thalassiosira* spp. *and Tintinnidae* were not well fit by the models and likewise had poor estimates of peak timing (Supplementary Materials Table 2).

Model 1 or Model 2 strongly described two of ten nutrients (Table 11), though neither model sufficiently explained the seasonal patterns for the other eight nutrients (Supplementary Table 1, Supplementary Figure 2). The seasonality of PO_4 was best fit by Model 2, explaining 66% of the deviance observed throughout the study, and the estimated peak timing was 253 ± 16. $NO_3 + NO_2$ was well fit by Model 1 and Model 2 and the estimated peak timing was earlier than PO_4 at day

200 ± 14. Water temperature, DO and salinity were also strongly seasonal (Table 11), whereas CHL, PHEO and turbidity had little to no seasonality based on poor fits to both models (Supplementary Table 1, Supplementary Figure 2). Water temperature was the most seasonal variable explored in this study, as 94% of the variability was fit by Model 2, and the estimated peak timing occurred at day 213 ± 2. The variances of DO and salinity were also well fit Model 2 (83% and 78% variability explained, respectively), though peak timing in these variables occurred at least ten days after water temperature (227 ± 7 and 248 ± 2, respectively).

Environmental variable correlation analysis

Overall, the plankton community, water quality conditions and the concentration of *Vibrio parahaemolyticus* in all matrices were highly correlated (Mantel test, $p = 0.008$). Univariate Spearman rank correlation analysis of the environmental variables provided an improved interpretability of these interactions, especially when a seasonality analysis was included since variables with similar strength of seasonality and peak timing were grouped together and significantly correlated (Figure 12). This seasonal grouping can be seen in the clustering and strength of correlation between *V. parahaemolyticus* in oysters, sediment and phytoplankton, as well as between these variables and *Helicotheca tamensis,* PO_4, salinity and water temperature. *V. parahaemolyticus* in water and *Chaetoceros* spp. also clustered with this group, though with lower strength of correlation. DO, $NO_3 + NO_2$, *Rhizosolenia* spp. and *Stephanopixis* spp. produced a separate cluster, but were also strongly inter-correlated and significantly negatively correlated with variables that clustered with *V. parahaemolyticus* in oysters. Significant correlative relationships and clustering was also seen between *V. parahaemolyticus* in zooplankton, photoperiod, nauplii and copepods. Additionally, significant correlation clusters were formed between seasonal plankton taxa and a separate cluster was formed by non-seasonal plankton taxa. Interestingly, no significant correlations were identified for plankton taxa or *V. parahaemolyticus* with CHL, PHEO, TSS, PN and PC. TDN and NPOC were only significantly correlated with *V. parahaemolyticus* in zooplankton.

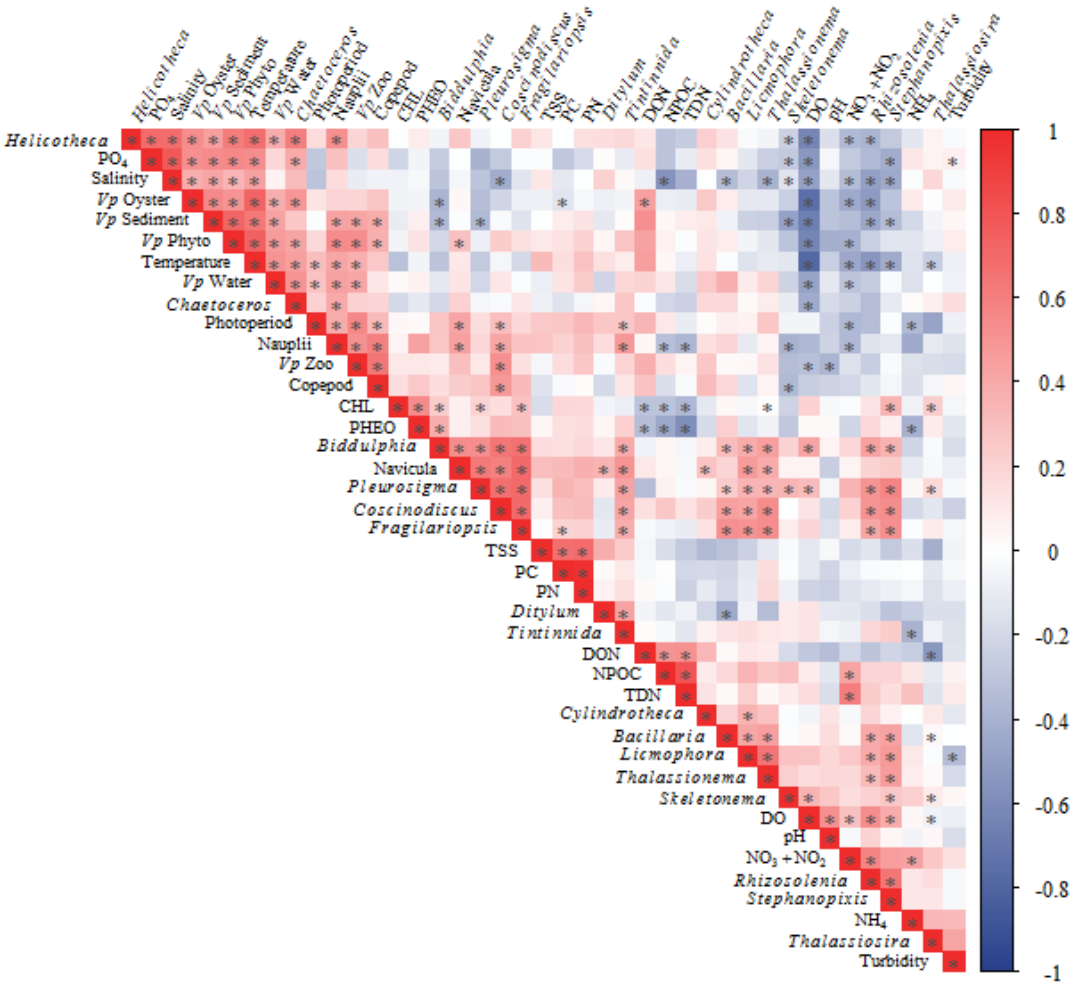


Figure 12. Spearman correlation analysis of *V. parahaemolyticus* in oysters, sediment, water and plankton, plankton taxa, water quality, nutrients. (*) indicates significant correlations.

Integrating seasonal variables to characterize the dynamics of V. parahaemolyticus concentrations in oysters

The relationships between the environmental variables and *Vibrio parahaemolyticus* dynamics in oysters were explored using integrative analysis with Partial Least Squared Residual modeling (PLSR). This multivariate analysis identified a subset of the environmental variables with an optimal one-factor solution to estimate *V. parahaemolyticus* dynamics in oysters including water temperature, salinity, dissolved oxygen, PO_4, *Chaetoceros* spp., *Helicotheca tamensis, Rhizosolenia* spp., and *V. parahaemolyticus* concentrations in sediment, phytoplankton and water (Table 12). The PLSR model using the entire suite of significant environmental variables (comprehensive) and the subset of variables from the comprehensive model with VIP > 0.8 (final) produced

similar RMSE (0.76) and estimations of variance, suggesting that *Biddulphia* spp., DON, NO_3 + NO_2 and PC did not provide unique information about the seasonal variance of *V. parahaemolyticus* concentrations in oysters.

Table 12. PLSR analysis of environmental variables for estimating *V. parahaemolyticus* concentrations in oysters.

Comprehensive			Final		
	PLSR 1	VIP		PLSR 1	VIP
Vp in Sediment	0.28	1.17	*Vp* in Sediment	0.28	0.88
Vp in Water	0.25	1.04	*Vp* in Water	0.27	0.76
Vp in Phytoplankton	0.26	1.08	*Vp* in Phytoplankton	0.24	0.86
Temperature	0.38	1.57	Temperature	0.40	1.26
Salinity	0.28	1.15	Salinity	0.27	0.85
DO	-0.34	1.42	DO	-0.36	1.15
PO_4	0.30	1.24	PO_4	0.34	1.06
Helicotheca	0.34	1.38	*Helicotheca*	0.36	1.13
Chaetoceros	0.25	1.05	*Chaetoceros*	0.27	0.85
Rhizosolenia	-0.30	1.24	*Rhizosolenia*	-0.34	1.09
Biddulphia	-0.12	0.27			
DON	0.15	0.61			
NO_3 + NO_2	-0.18	0.78			
PC	-0.16	0.52			
Cumulative Variance	0.49		Cumulative Variance	0.48	
RMSEP	0.76		RMSEP	0.76	

Structural equation modeling

Correlative relationships between the variables identified as significant by PLSR were assessed to inform the development of SEM (Table 13). Due to the strength of the signal of inter-correlation, individual SEM models were developed in a stepwise manner for: 1.) *V. parahaemolyticus* in sediment, water and phytoplankton (*Vp* model), then incorporated with 2.) plankton taxa (biotic model) and finally 3.) water quality variables (abiotic model) before combining them to produce a holistic overview of the seasonal ecology as it relates to *V. parahaemolyticus* concentrations in oysters (Table 14).

Table 13. Correlations between seasonally driven *V. parahaemolyticus* concentrations in sediment, water and phytoplankton, plankton species, water quality and nutrients.

	Vp Sediment	*Vp* Water	*Vp* Phyto	PO_4	*Chaetoceros* spp.	*Helicotheca Tamensis*	*Rhizosolenia* spp.	Temperature	Salinity	Dissolved Oxygen
Vp Oyster	0.55	0.38	0.51	0.52	0.49	0.54	-0.52	0.73	0.39	-0.74
Vp Sediment		0.44	0.73	0.55	0.26	0.45	-0.45	0.63	0.42	-0.66
Vp Water			0.48	0.19	0.50	0.38	-0.27	0.50	0.07	-0.48
Vp Phyto				0.55	0.43	0.62	-0.25	0.74	0.34	-0.63
PO_4					0.36	0.68	-0.31	0.51	0.79	-0.53
Chaetoceros spp.						0.60	-0.38	0.46	0.24	-0.45
Helicotheca tamensis							-0.40	0.69	0.63	-0.66
Rhizosolenia spp.								-0.57	-0.40	0.55
Temperature									0.46	-0.88
Salinity										-0.58

Table 14. Stepwise Structural Equation Model development using *V. parahaemolyticus* in sediment, water and phytoplankton (*Vp*), plankton species (Biotic), water quality and nutrients (Abiotic) and all factors (Abiotic and Biotic).

	df	$\chi 2$	Comparative Fit	Tucker Lewis Fit	Δ Likelihood	AIC	SRMR	r^2
Vp	6	0.649	1	1.46	-0.15	258.9	0.024	0.22
Biotic	6	0.415	0.998	0.995	-3.21	368.3	0.101	0.40
Abiotic	10	0.577	1	1.06	-0.57	268.93	0.044	0.47
Abiotic + Biotic	28	0.22	0.987	0.882	-2.2	324.2	0.045	0.57

Vibrio parahaemolyticus concentrations in sediment provided the best estimation of *V. parahaemolyticus* concentrations in oysters (r^2=0.32, p = 0.0002, AIC = 179.6), followed by *V. parahaemolyticus* associated with phytoplankton (r^2 = 0.22, p = 0.002, AIC = 184.7) in the *Vp* model. *V. parahaemolyticus* concentrations in sediment and phytoplankton covaried significantly (R = 0.73, p = <0.0001). *V. parahaemolyticus* in water provided the lowest estimation of *V. parahaemolyticus* concentrations in oysters (r^2=0.11, p = 0.032 AIC = 198.3). The final *Vp* model included *Vibrio parahaemolyticus* concentrations in sediment and in phytoplankton, and explained 22% of the variance observed for *V. parahaemolyticus* concentrations in oysters.

The biotic model, that incorporated plankton taxa with the *Vp* model, identified that *Helicotheca tamensis* significantly covaried with *Vibrio parahaemolyticus* concentrations in oysters and phytoplankton whereas *Rhizosolenia* spp. significantly covaried with *V. parahaemolyticus* concentrations in oysters and sediment. *Chaetoceros* spp. did not significantly covary with *V. parahaemolyticus* concentrations in oysters, sediment or phytoplankton and was not included in the final biotic model. The addition of the plankton taxa to the *Vp* model improved the estimation of *V. parahaemolyticus* concentrations in oysters from 22% to 40%. In the development of the abiotic model, water temperature alone estimated 43.6% of the variance in *V. parahaemolyticus* concentration in oysters. It was determined that water temperature was also correlated with the variance in DO, salinity and PO_4. An interaction between salinity and PO_4 was accounted for in the final abiotic and abiotic + biotic SEM.

The biotic and abiotic model combined produced a holistic SEM that accounted for the complex combinations of abiotic and biotic variables that relate to the dynamics *Vibrio parahaemolyticus* dynamics in oysters. Together, 57% of the variance observed in oysters was best fit by a combination of *V. parahaemolyticus* concentration in sediment and phytoplankton, *Helicotheca tamensis* and *Rhizosolenia* spp. concentrations, water temperature, PO_4 and salinity ($\chi^2 = 0.22$, $r^2 = 0.55$). *V. parahaemolyticus* concentration in water, *Chaetoceros* spp. and DO did not contribute to the SEM model to explain *V. parahaemolyticus* dynamics in oysters and were not included in the final model. Overall, water temperature had the strongest correlation to *V. parahaemolyticus* concentrations in oysters (Figure 13). Water temperature was also significantly correlated with the other variables in the abiotic and biotic models, though the strength of this correlation varied. The variance of *V. parahaemolyticus* concentrations in sediment and phytoplankton were both strongly correlated with *V. parahaemolyticus* concentration variation in oysters, though sediment was stronger overall. *Helicotheca tamensis* and *Rhizosolenia* spp. improved model fit significantly by fitting additional variance of *V. parahaemolyticus* concentrations. PO_4 provided a stronger fit to the model than salinity, however salinity was retained in the final SEM because of its contribution to the variance of PO_4. Substantial inter-correlation was also observed between plankton taxa, water quality variables and *V. parahaemolyticus* in sediment and oysters.

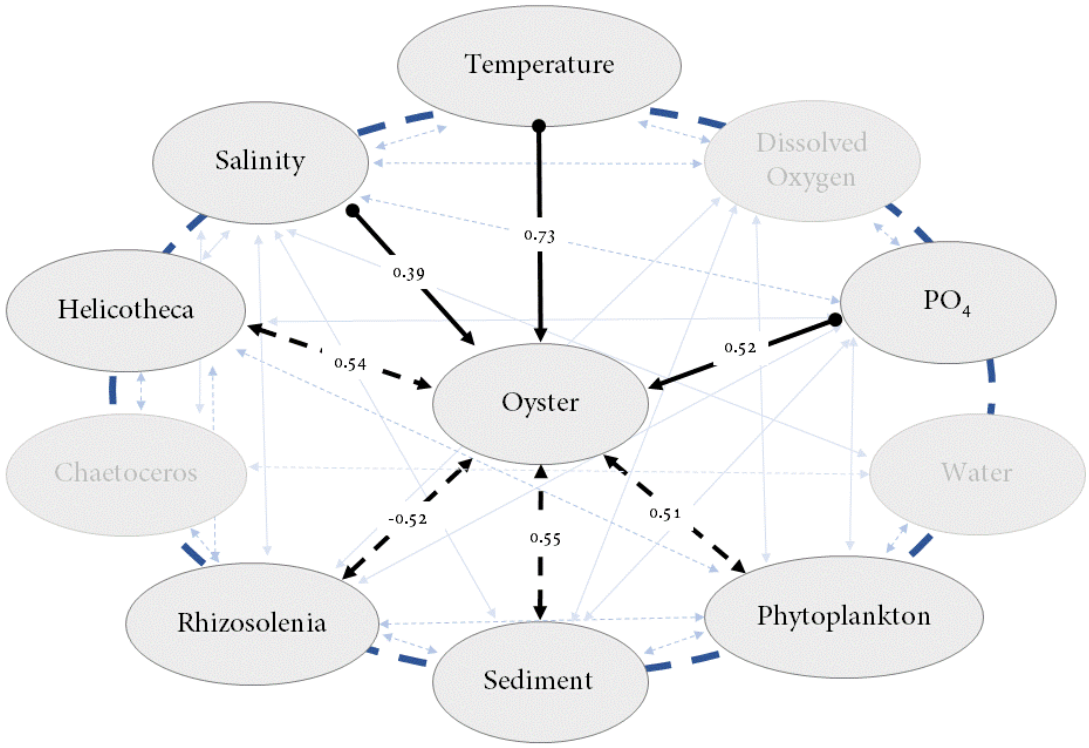


Figure 13. Correlative and intercorrelated relationships between *V. parahaemolyticus* in oysters and seasonal biotic and abiotic variables. Correlative relationships that are significant to the estimation of *V. parahaemolyticus* concentration in oysters is shown in black. Water temperature was significantly related to all variables and this is indicated by the dashed blue line. Thin blue lines indicating significant regressions between abiotic and biotic parameters are solid whereas significant covariances between biotic variables are dashed. Variables that were not significant to the estimation of *V. parahaemolyticus* concentrations in oysters are light grey.

These inter-correlations between the environmental variables provided important additional insight into their importance to the final SEM to estimate *V. parahaemolyticus* concentrations in oysters. For example, the contribution of *Helicotheca tamensis* and *Rhizosolenia* spp. can be better understood in light of their importance to explaining the variability of estimating *V. parahaemolyticus* concentrations in phytoplankton and in sediment, respectively. PO_4 strongly estimated *Helicotheca tamensis* and was significant to *V. parahaemolyticus* concentrations in oysters, sediment and phytoplankton.

The potential contribution of each variable to the dynamics of *Vibrio parahaemolyticus* concentrations in oysters begins to emerge at an improved resolution in the context of seasonality.

For example, water temperature was the strongest estimator of *V. parahaemolyticus* concentrations in oysters and is also highly correlated with other abiotic and biotic variables. It is also the most seasonally driven variable and its seasonal peak timing occurs first, compared to all other variables, at day 213± 2 (Fig. 13). The peak timing of the remaining biotic and abiotic variables in the model then occur roughly within three weeks following the peak water temperature with varying strengths of seasonality. Peak timing of *Helicotheca tamensis* and *Rhizosolenia* spp. occurs approximately one week of after the peak timing of water temperature at around the same peak timing for *V. parahaemolyticus* concentrations in phytoplankton. The peak timing of *V. parahaemolyticus* concentrations in oysters was at day 230 ± 13 and was followed by the peak for *V. parahaemolyticus* concentrations in sediment. *V. parahaemolyticus* concentrations in sediment had a pronounced fall 'shoulder', i.e., a delayed decreasing trend during fall, in contrast to earlier peak timing and a pronounced decrease during fall for *V. parahaemolyticus* concentrations in oysters or phytoplankton (Fig. 13f). Finally, peak salinity and PO_4 are estimated to occur in early September, approximately at the same time as *V. parahaemolyticus* in sediment, though the CI for PO_4 is more pronounced than salinity.

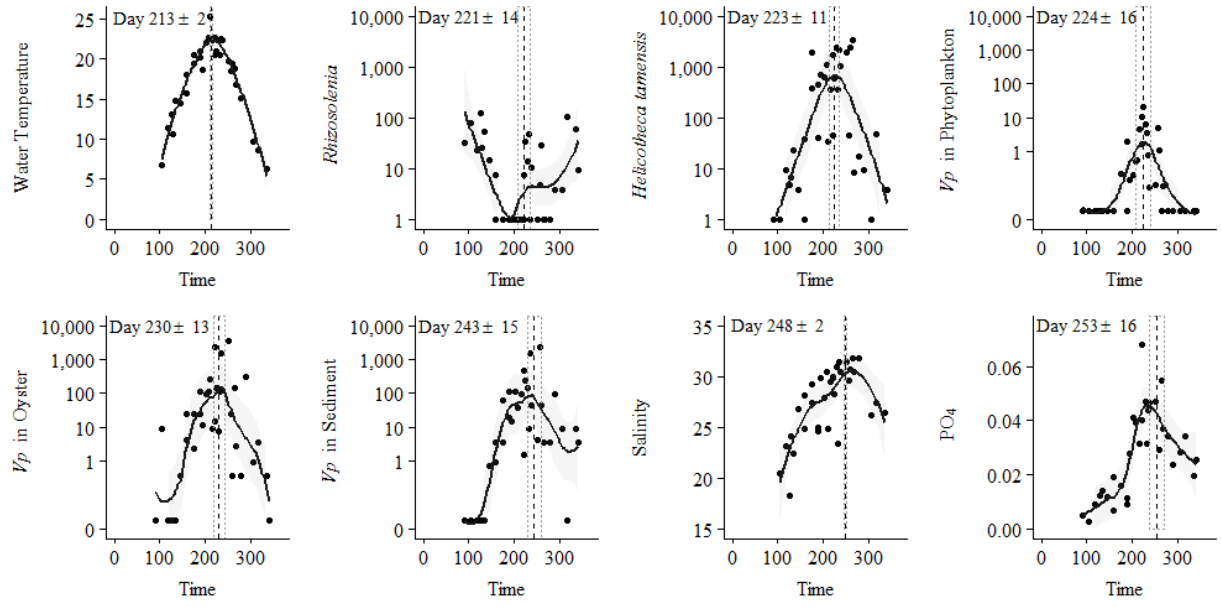


Fig 14. Seasonality and peak timing of (a) water temperature, (b) *Rhizosolenia*, (c) *Helicotheca tamensis*, (d) *V. parahaemolyticus* concentrations in phytoplankton, (e) *V. parahaemolyticus* in oysters, (f) *V. parahaemolyticus* in sediment, (g) salinity, and (h) PO_4, superimposed by year and overlaid with loess smoothing.

Together, the correlations, inter-correlations and patterns of seasonality in the environmental variables considered provide important insight into the seasonal microbial ecology of the GBE and illustrates how the combined influence of each variable in light of seasonal timing and correlative strength may contribute to the overall seasonality of *Vibrio parahaemolyticus* concentration in oysters.

DISCUSSION

The emergence and establishment of *Vibrio parahaemolyticus* as a causative agent of shellfish-borne illnesses in the Northeast United States has stimulated keen interest in understanding the coastal ecosystem conditions that may influence this human health risk in this region. As *V. parahaemolyticus* disease dynamics are changing worldwide, plankton dynamics, including the distribution of plankton taxa and the frequency and intensity of harmful algal blooms, are also changing concurrent with climate related changes in the marine environment (Cavicchioli et al., 2019; Townhill et al., 2018; Baker-Austin et al., 2017; Grimes et al., 2014; Vezzulli et al., 2013). Recent work based on a more limited set of environmental variables determined that *V. parahaemolyticus* dynamics in the GBE are strongly influenced by seasonality and that patterns of this seasonality are changing over time as *V. parahaemolyticus* concentrations are increasingly elevated throughout the summer and are remaining high later into fall months (Hartwick et al., 2019). Using an expanded suite of water quality and biological factors, we determined that *V. parahaemolyticus* concentrations in sediment and phytoplankton, *Helicotheca tamensis*, *Rhizosolenia* spp., water temperature, salinity and PO_4 in the GBE are also seasonally driven in the GBE and applied integrative multivariate analysis to illustrate how their seasonal dynamics may ultimately contribute to the accumulation *V. parahaemolyticus* concentration in oysters. This improved resolution of the environmental variables that associate with *V. parahaemolyticus* in the GBE provides a basis to identify the environmental variables that contribute to the seasonality of *V. parahaemolyticus* concentrations and to potentially better characterize the conditions that may contribute to associate with human health risks in this region.

In the Northeast US, *Vibrio parahaemolyticus* concentrations and water temperature are highly seasonal and *V. parahaemolyticus* dynamics most strongly correlate with water temperature as

reported for other temperate regions (Oberbeckman et al., 2011; Deter et al., 2010). In other regions where *V. parahaemolyticus* dynamics are also seasonal yet driven by other environmental conditions such as monsoons season, the relationship between temperature and *V. parahaemolyticus* is not significant (Deepanjali et al., 2005). In New Hampshire, seasonality may be one probable explanation for why regression modeling favors water temperature as the most significant model parameter, among others (dissolved oxygen, pH, salinity and nutrients including: $NO_3 + NO_2$, TDN and PO_4) in the GBE, for estimating *V. parahaemolyticus* concentrations in univariate regression. A complex combination of biological and physical environmental variables certainly drives *V. parahaemolyticus* population dynamics, however, many of these environmental variables are, in turn, driven mainly by seasonal temperature and therefore do not contribute unique variance to a statistical model for describing seasonal ecology.

In this study, water temperature estimated 43.6% of the observed variability in *V. parahaemolyticus* concentrations in oysters, and both the association with water temperature and the differing patterns of seasonality varied between *V. parahaemolyticus* concentrations in oysters, sediment, water, phyto and zooplankton. The influence of covarying environmental variables in statistical models is likely one of many reasons why a complex array of environment variables has been observed in studies around the world to correlate with *Vibrio parahaemolyticus* dynamics, though other factors certainly relate to this, including differences between study design and analytic methods. Differences in *V. parahaemolyticus* dynamics between matrices have been frequently observed and some studies have applied models comprised of different environmental variables to estimate *V. parahaemolyticus* in varying matrices (Nilsson et al., 2019, Deter et al., 2010). In the GBE, the initial detection of *V. parahaemolyticus* in oysters and sediment began early in the year and lasted through late fall though the peak timing of *V. parahaemolyticus* concentration dynamics differed between matrices in terms of onset of detection and peak timing. The relationships between matrices varied in significance, suggesting that some were more useful for explaining *V. parahaemolyticus* concentrations in oysters.

There were similarities in the dynamics of *Vibrio parahaemolyticus* between matrices. For example, *V. parahaemolyticus* concentrations in oysters were most strongly associated with *V.*

parahaemolyticus concentrations in sediment although there was also strong inter-correlation between *V. parahaemolyticus* concentrations in sediment and phytoplankton. Though the importance of the host shellfish to *V. parahaemolyticus* persistence is not clear (Lovell et al., 2017; DePaola et al. 1990, 2003; Zimmerman et al. 2007), the accumulation of *V. parahaemolyticus* via filter feeding of suspended sediments and plankton species has been frequently suggested (e.g., Froelich et al. 2013, Kaneko and Colwell et al., 1973, 1975). To account for these differences, we applied seasonality analysis and peak timing to assess each variable for their contribution to the overall seasonal ecology of *V. parahaemolyticus* in oysters in the GBE. For example, although *V. parahaemolyticus* concentrations in sediment have the strongest correlative relationship to *V. parahaemolyticus* concentrations in oysters, given the dynamics of sediment, diatoms on sediments and in the water column, and the peak timing of *V. parahaemolyticus* in phytoplankton, phytoplankton are also probably an important influence, perhaps in different ways or at different times, on the seasonal pattern of *V. parahaemolyticus* in oysters. To further this point, *V. parahaemolyticus* concentration in oysters peak within a week of *V. parahaemolyticus* concentrations in phytoplankton whereas the pattern of seasonal emergence and gradually decreasing concentrations in the fall is more similar between *V. parahaemolyticus* in sediment and oysters.

The variable temporal dynamics observed in *Vibrio parahaemolyticus* concentrations were similar to the temporal dynamics of the GBE plankton community, where the patterns of community assemblage tracked closely with water temperature, though the overall plankton abundance did not. This was due in large part to individual taxa such as *Skeletonema* spp. that were present in high numbers in early spring, or *Fragilariopsis* spp. and *Navicula* spp. that were present year-round. Though *Skeletonema spp.* have also been reported as the major component of other temperate water systems, it is also observed globally in marine systems (Canesi and Rynearson, 2016). Likewise, *Fragilariopsis* spp. has been reported in many locations around the world, though it is generally associated with cold-water regions (Mock et al., 2017).

Helicotheca tamensis was significantly positively associated with *V. parahaemolyticus* dynamics in oysters in the GBE. In the GBE it was often seen in single cell or short, straight square chains, though it has been reported to form extended, twisted chains as well (Hernández-Becerril et al.,

2013). The ecology of *H. tamensis* is not well characterized, however it has been detected in a wide range of environments including: the Bay of Fundy, the Baltic Sea, the Gulf of Mexico and the Port of Recife in Brazil (Santiago et al., 2010, Halifors et al., 2004, Martin et al., 2001). In the Port of Recife, which is classified as a eutrophic estuary, *H. tamensis* can dominate the plankton community in summer months. The GBE is not considered a eutrophic estuary but *H. tamensis* abundance can exceed >75% of the plankton community in late August and September, however never at the densities seen elsewhere. Gilbert et al., (2012) and Furhman et al., (2008) suggests that plankton community assemblage can be been attributed to a combination of factors, strongly driven by water temperature, but modulated by individual temporal niches related to water quality, nutrients or meteorological conditions that enable complex community dynamics in temperate regions, similar to what is found for *V. parahaemolyticus* in our and other studies. The co-occurring summer dynamics of *H. tamensis* and *V. parahaemolyticus* in oysters may indicate that they share similar niche or physiological preferences, though these would require a more in-depth study to fully characterize. Alternatively, *H. tamensis* abundance in the GBE could serve as an easily measurable indicator of *V. parahaemolyticus* abundance in oysters in later summer and early fall. It is also important to note that previous studies of the plankton taxa present in the GBE do not mention *H. tamensis* (Jones, 2000; NAI, 1985). This could mean that *H. tamensis* is a recent addition to the GBE plankton community and that the microbial community is being reshaped via introduced species or changing ecological conditions.

Very little recent data on plankton communities and their dynamics in the GBE were available prior to this study, so this study also provides an important update for this area. The plankton community in the GBE appears to be diatom-dominated, as previously reported (Jones, 2000; NAI, 1985). Though plankton species were detected in the water column from April through December, on average the abundance was 6,700 cells/L and this varied strongly by time of the year. Elevated concentrations from 10,000 to 35,000 cells/L were only seen in seven samples, which were also those dominated by *Cheatocerus* spp. (n=5), *Navicula* spp. (n=1) or *Skeletonema* spp. (n=1). Similarly, the concentration of chlorophyll-*a* was also generally low, with an average of 6.3±4.5 μg/L. Together these findings suggest that the GBE has potentially limited water column primary productivity and lack of relationships between *Vibrio parahaemolyticus* in oysters

with total abundance or the most abundant plankton taxa suggests that the use of plankton taxa that do relate to *V. parahaemolyticus* dynamics would be better monitoring tools than chlorophyll-*a*.

Chlorophyll-*a* is a widely used monitoring variable related to primary productivity in coastal waters and, along with individual phytoplankton taxa, is a potentially useful tool for characterizing risks associated with *Vibrio parahaemolyticus* in shellfish. There was also no correlation found between chlorophyll-a and *V. parahaemolyticus* dynamics in the GBE. The absence of a correlative relationship between chlorophyll-*a* and either *V. parahaemolyticus* concentrations or phytoplankton dynamics found in this study has been reported elsewhere (Takemura et al., 2014), suggesting again that phytoplankton taxa may be better monitoring targets for tracking *V. parahaemolyticus* dynamics. Rhenstam-Holm et al. (2010) also observed a negative relationship between *Skeletonema* spp. and *Chaetoceros* spp. blooms and *V. parahaemolyticus* concentrations in their 2010 study, though Gilbert et al. (2012) identified an incidental co-occurring bloom of *V. parahaemolyticus* and *Chaetoceros* spp. during their study suggesting a positive bacterio-plankton interaction. There is work that has shown that *V. parahaemolyticus* can also support a free-living lifestyle in the water column by subsisting off nutrient-rich floccula or polysaccharide exudate (Takemura et al., 2014; Gilbert et al., 2010; Venkataswaran, 1990) that may provide insight into why *V. parahaemolyticus* dynamics are not related to chlorophyll-*a* or observed plankton blooms in the GBE. Given the relative low plankton abundance observed in this study, it is important to consider that alternate factors might be more strongly related to *V. parahaemolyticus* dynamics in the GBE, despite the reported importance of phytoplankton blooms or chlorophyll-*a* reported elsewhere. In addition, the lack of a correlative relationship between chlorophyll-*a* and overall plankton abundance or individual plankton taxa identified in this study suggests alternate variables to chlorophyll-*a* should be considered to monitor plankton dynamics in the GBE, at least at the size fraction of >53µm.

Nutrients are often monitored in coastal waters for many reasons and are potentially influential variables that relate to both *Vibrio parahaemolyticus* and plankton dynamics (Paranjpye et al., 2015). The contribution of nutrients to plankton and *V. parahaemolyticus* dynamics was also considered

as nutrient availability is likely one of the most important limiting factors of *V. parahaemolyticus* dynamics in light of the metabolic demands based on its rapid growth rate (Espejo et al., 2017, Turner et al., 2016, Johnson et al., 2013). The majority of nutrient variables were not seasonal or significantly correlated to *V. parahaemolyticus* in oysters, sediment, plankton, and water or to individual plankton taxa. The exception to this was NO_3 + NO_2 and PO_4, both of which were highly seasonal and strongly correlated with *V. parahaemolyticus* overall, and PO_4 was determined to be one of the most important variables to estimate *V. parahaemolyticus* in oysters in multivariate analysis. An earlier study of *V. parahaemolyticus* concentrations in GBE water also reported PO_4 as a significant variable, in addition to water temperature, total and organic suspended solids (Jones and Summer-Brason, 1998). Though the majority of nutrients were not significant to plankton or *V. parahaemolyticus* dynamics in this study, previous studies (Blackwell and Oliver, 2008, Oberbeckman et al., 2011) have reported positive correlations between *Vibrio spp.* and nutrients such as TDN. It should be considered that the dynamics between nutrients, plankton and *V. parahaemolyticus* are complex, and a lack of significance here could relate to some dimension of these dynamics that was not accounted for in this study like the timing of nutrient loading events and both plankton blooms and their decline (Trombetta et al., 2019; Cloern, 1996). For example, low nutrient conditions may reflect the onset of declines in plankton blooms that can then cause release of nutrients and polysaccharides from decaying plankton cells (Mühlenbruch et al., 2018). A lack of significance between nutrient and *V. parahaemolyticus* concentrations could also reflect the rapid turnover in nutrients during warm summer conditions and because their influence on *V. parahaemolyticus* concentrations is an indirect interaction that does not consider the more direct effect of nutrients on the plankton community (Gude, 1985). Future work with more frequent sampling could provide an improved resolution of the contribution of nutrients to the microbial community dynamics in the GBE.

Peak timing was used to assess the environmental variables in this study to determine how they may individually contribute to the development of ideal conditions for *Vibrio parahaemolyticus.* We determined that significant predictive variables peak both in advance and following the peak timing of *V. parahaemolyticus* in oysters at day 230 (± 13). The photoperiod variable peaks first, on the longest day of the year (day 170). This is followed by the peak timing of water temperature

(213 ± 2), then summer indicator phytoplankton *Rhizosolenia* spp. and *Helicotheca tamensis*, and *V. parahaemolyticus* in phytoplankton, all potentially contributing to a hysteresis or loading of the system that sets up optimal conditions for *V. parahaemolyticus* in oysters. Interestingly, especially in relationship to filter feeding dynamics, the concentration of *V. parahaemolyticus* in the water was poorly correlated with *V. parahaemolyticus* in oysters and sediment. It's worth noting however, that *Chaetoceros* spp. was the strongest estimator of *V. parahaemolyticus* in water. So, although *Chaetoceros* spp. were the most abundant plankton taxa in the GBE in this study, they did not strongly correlate with *V. parahaemolyticus* in oysters, suggesting *Chaetoceros* spp. may have some undefined relation to overall *V. parahaemolyticus* dynamics in the full suite of ecosystem matrices that exist in the study area. Finally, *V. parahaemolyticus* in sediment peak timing occurs on day 243 ± 15 followed by salinity and PO_4 and though the peak timing of *V. parahaemolyticus* in oysters occurs ~2 weeks prior, elevated concentrations are still observed during this time, suggesting that *V. parahaemolyticus* associated with sediments may be a key late summer driver of the dynamics of oyster *V. parahaemolyticus* concentrations.

Previous modeling efforts in the GBE determined that the characteristics of seasonality combined with water temperature, pH, salinity and photoperiod improved the estimation of *Vibrio parahaemolyticus* incidence and concentration predictions in the GBE (Hartwick et al., 2019; Urquhart et al., 2016). The relationship with photoperiod and community dynamics in phytoplankton and *V. parahaemolyticus* was also observed by Gilbert et al. (2012) and Nilsson et al. (2019) in other coastal areas. Moreover, the importance of lags upwards of a month to effectively estimate *V. parahaemolyticus* concentrations was recently shown by Davis et al., (2019). Data in this study were collected either monthly or biweekly, whereas *V. parahaemolyticus* can replicate every eight minutes under ideal conditions. Traditional methods for lag analysis and cross correlation could provide an improved resolution of these lagged effects on *V. parahaemolyticus* with more frequent sampling and fine-scale temporal resolution. However, in this study, the application of harmonic regression and peak timing demonstrates how biological complexities and limitations of sampling frequency can be accounted for and provide an in-depth characterization of the ecological dynamics of plankton and *V. parahaemolyticus* in a highly seasonal estuary.

CONCLUSIONS

The seasonality of an extended suite of water quality, meteorological conditions and biological community variables was assessed to characterize the interactions of plankton and *Vibrio parahaemolyticus* dynamics oysters in the GBE. The overall dynamics of *V. parahaemolyticus* were highly seasonal and patterns of peak timing and the onset of detection varied considerably, suggesting complex ecological linkages between co-occurring plankton, water quality and *V. parahaemolyticus* dynamics. The relationships observed between plankton community abundance, blooms and *V. parahaemolyticus* concentrations in phyto- and zooplankton suggests that indicators such as chlorophyll-*a* and plankton blooms may not be informative leading indicators of *V. parahaemolyticus* dynamics in oysters in the GBE. However, the shared seasonality of water temperature, salinity, PO_4, *Helicotheca tamensis*, and *V. parahaemolyticus* in sediment and phytoplankton provides a first step towards a more in-depth description of the biotic and abiotic ecology of *V. parahaemolyticus*. Though a complex combination of environmental variables certainly drives *V. parahaemolyticus* dynamics, including location specific ecology and potentially population specific genetics/genomics (Froelich and Noble, 2016; Shapiro and Polz, 2014), this work provides a first step towards the characterization of how plankton and other environmental variables contribute to the overarching seasonality and are drivers that may be contributing to the changing dynamics of *V. parahaemolyticus* in oysters in the Northeast US. The overall analytical approach is a useful and potentially transferable framework for similar studies in other temperate coastal areas.

Supplementary Materials: Figure 1: Seasonal Environmental variables and peak timing. Figure 2: Environmental variables with limited seasonal periodicity. Table S1: Environmental Variables with minimal seasonality based on photoperiod and harmonic regression modeling.

Author Contributions: Conceptualization, MAH, AB, ENN and SHJ.; methodology, MAH, NL, AB and SHJ.; software, MAH, AB.; validation, CAW, VSC, ENN and SHJ.; formal analysis, MAH; resources, CAW, VSC, SHJ.; data curation, MAH and AB.; writing—original MAH, AB; writing—review and editing, CAW, VSC, ENN and SHJ.; visualization, MAH and AB.; supervision, SJH.; funding acquisition, CAW, VSC, SHJ.

Funding: The authors gratefully acknowledge partial funding support from the National Science Foundation EPSCoR IIA-1330641, USDA National Institute of Food and Agriculture Hatch NH00574, NH00609 (accession 233555), and NH00625 (accession 1004199), and the National Oceanic and Atmospheric Administration College Sea Grant program and New Hampshire Sea Grant program grants R/CE-137, R/SSS-2, R/HCE- 3

Acknowledgments: The authors would like to thank Jennifer Mahoney, Meg Striplin, Brian Schuster, Crystal Ellis, Jong Yu, Eliot Jones, Michael Taylor, Ashley Marcinkiewicz, Feng Xu, Tom Gregory, Chris Peters, Jackie Lemaire, Sarah Richards, Emily Schulz, and Elizabeth Deyett for their help with sampling, sample processing, detection analysis, and database management. Also, Alexandra Kulinkina and Tania M. Alarcon Falconi for support with implementing harmonic regression analysis and peak timing calculations in the R environment.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

Hartwick, M. A., Urquhart, E. A., Whistler, C. A., Cooper, V. S., Naumova, E. N., & Jones, S. H. (2019). Forecasting Seasonal *Vibrio parahaemolyticus* Concentrations in New England Shellfish. International journal of environmental research and public health, 16(22), 4341.

Lovell, C. R. Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Applied microbiology and biotechnology* **2017**, 101(5), 1781-1794.

Urquhart, E. A.; Jones, S. H.; Jong, W. Y.; Schuster, B. M.; Marcinkiewicz, A. L.; Whistler, C. A.; Cooper, V. S. Environmental conditions associated with elevated *Vibrio parahaemolyticus* concentrations in Great Bay Estuary, New Hampshire. *PloS one* **2016**, *11*(5), e0155018.

Turner, J. W.; Malayil, L.; Guadagnoli, D.; Cole, D.; Lipp, E. K. Detection of *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae* with respect to seasonal fluctuations in temperature and plankton abundance. *Environmental microbiology* **2014,** 16(4), 1019-1028.

Martinez-Urtaza, J.; Blanco-Abad, V.; Rodriguez-Castro, A.; Ansede-Bermejo, J.; Miranda, A.; Rodriguez-Alvarez, M. X.; Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus in* offshore areas. *The ISME Journal* **2012**, *6*(5), 994–1006.

Vezzulli, L.; Pezzati, E.; Moreno, M.; Fabiano, M.; Pane, L.; Pruzzo, C.; VibrioSea Consortium. Benthic ecology of *Vibrio* spp. and pathogenic Vibrio species in a coastal Mediterranean environment (La Spezia Gulf, Italy). *Microbial ecology* **2009,** *58*(4), 808-818.

Jones, S.; Summer-Brason, B. Incidence and detection of pathogenic *Vibrio* spp. in a northern New England estuary, USA. *Journal of Shellfish Research* **1998,** *17*, 1665–1669.

DePaola, A.; Hopkins, L.H.; Peeler, J.T.; Wentz, B.; McPhearson, R.M. *Incidence of Vibrio parahaemolyticus* in US coastal waters and oysters. *Appl. Environ. Microbiol*. **1990,** *1;56(8*), 2299-302.

Kaneko, T., & Colwell, R. R. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Journal of Bacteriology 1973, 113(1), 24-32

Erken M, Lutz C, McDougald D. 2015. Interactions of *Vibrio* spp. with zooplankton. microbiolspec 3(3): doi:10.1128/microbiolspec.VE-0003-2014

Vezzulli, L., Colwell, R. R., & Pruzzo, C. Ocean warming and spread of pathogenic *Vibrios* in the aquatic environment. *Microbial ecology* **2013**, 65(4), 817-825.

Hughes, S. N., Greig, D. J., Miller, W. A., Byrne, B. A., Gulland, F. M., & Harvey, J. T. (2013). Dynamics of Vibrio with virulence genes detected in Pacific harbor seals (Phoca vitulina richardii) off California: implications for marine mammal health. Microbial ecology, 65(4), 982-994.

Martinez-Urtaza, J.; Lozano-Leon, A.; Varela-Pet, J.; Trinanes, J.; Pazos, Y.; Garcia-Martin, O. Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the rias of Galicia, Spain. *Appl. Environ. Microbiol.* **2008**, *74*(1), 265-274.

Alipour, M., Issazadeh, K., & Soleimani, J. (2014). Isolation and identification of *Vibrio parahaemolyticus* from seawater and sediment samples in the southern coast of the Caspian Sea. Comparative clinical pathology, 23(1), 129-133.

Williams, T. C., Ayrapetyan, M., & Oliver, J. D. (2014). Implications of chitin attachment for the environmental persistence and clinical nature of the human pathogen *Vibrio vulnificus*. Appl. Environ. Microbiol., 80(5), 1580-1587.

Froelich, B., Ayrapetyan, M., & Oliver, J. D. (2013). Integration of *Vibrio vulnificus* into marine aggregates and its subsequent uptake by *Crassostrea virginica* oysters. Appl. Environ. Microbiol., 79(5), 1454-1458.

Parveen, S.; Hettiarachchi, K.A.; Bowers, J.C.; Jones, J.L.; Tamplin, M.L.; McKay, R.; Beatty, W.; Brohawn, K.; DaSilva, L.V.; DePaola, A. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International journal of food microbiology* **2008**, *128*(2), 354-361.

DePaola, A.; Nordstrom, J.L.; Bowers, J.C.; Wells, J.G.; Cook, D.W. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl. Environ. Microbiol. 2003, 69:1521–1526.

Johnson, C. N. (2013). Fitness factors in vibrios: a mini-review. Microbial ecology, 65(4), 826-851.

Zimmerman, A. M.; DePaola, A.; Bowers, J. C.; Krantz, J. A.; Nordstrom, J. L.; Johnson, C. N.; Grimes, D. J. Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Mexico water and oysters. Appl. Environ. Microbiol **2007,** *73(23*), 7589-7596.

Kaneko, T., & Colwell, R. R. (1977). The annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. Microbial ecology, 4(2), 135-155.

Caburlotto, G.; Haley, B. J.; Lleò, M. M.; Huq, A.; Colwell, R. R. Serodiversity and ecological distribution of *Vibrio parahaemolyticus* in the Venetian Lagoon, Northeast Italy. *Environmental microbiology reports* **2010,** *2(1*), 151-157.

Takemura, A.; Chien, D.; Polz, M. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology* **2014**, *5*(38).

Turner, J. W.; Good, B.; Cole, D.; Lipp, E. K. Plankton composition and environmental factors contribute to Vibrio seasonality. *The ISME journal* **2009**, *3(9*), 1082.

Paranjpye, R. N.; Nilsson, W. B.; Liermann, M.; Hilborn, E.D.; George, B.J.; Li, Q.; Bill, B.D.; Trainer, V.L.; Strom, M.S.; Sandifer, P.A. Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA. FEMS microbiology ecology 2015, 91(12), 121.

Gilbert, J.A.; Steele, J.A.; Caporaso, J.G.; Steinbrück, L.; Reeder, J.; Temperton, B.; Huse, S.; McHardy, A.C.; Knight, R.; Joint, I.; Somerfield, P. Defining seasonal marine microbial community dynamics. The ISME journal 2012, 6(2), p.298.

Grimes, D. J.; Ford, T. E.; Colwell, R. R.; Baker-Austin, C.; Martinez-Urtaza, J.; Subramaniam, A.; Capone, D. G. Viewing marine bacteria, their activity and response to environmental drivers from orbit: satellite remote sensing of bacteria. *Microbial Ecology* **2014,** *67*(3), 489–500.

Asplund, Maria E., Ann-Sofi Rehnstam-Holm, Vijay Atnur, Pendru Raghunath, Vasudevan Saravanan, Karolina Härnström, Betty Collin, Indrani Karunasagar, and Anna Godhe. "Water column dynamics of Vibrio in relation to phytoplankton community composition and environmental conditions in a tropical coastal area." Environmental microbiology 13, no. 10 (2011): 2738-2751.

Beveridge, O. S., Petchey, O. L., & Humphries, S. (2010). Direct and indirect effects of temperature on the population dynamics and ecosystem functioning of aquatic microbial ecosystems. Journal of Animal Ecology, 79(6), 1324-1331.

Kaneko, T., & Colwell, R. R. (1975). Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Environ. Microbiol., 30(2), 251-257.

Cavicchioli, R., Ripple, W.J., Timmis, K.N., Azam, F., Bakken, L.R., Baylis, M., Behrenfeld, M.J., Boetius, A., Boyd, P.W., Classen, A.T., Crowther, T.W., Danovaro, R., Foreman, C.M., Huisman, J., Hutchins, D.A., Jansson, J.K., Karl, D.M., Koskella, B., Welch, M.D.B., Martiny, J.B.H., Moran, M.A., Orphan, V.J., Reay, D.S., Remais, J.V., Rich, V.I., Singh, B.K., Stein, L.Y., Stewart, F.J., Sullivan, M.B., van Oppen, M.J.H., Weaver, S.C., Webb, E.A. Webster, N.S. Scientists' warning to humanity: microorganisms and climate change, Nature Reviews Microbiology, (2019) 569,586,17; 9. https://doi.org/10.1038/s41579-019-0222-5

Townhill, B.L., Tinker, J., Jones, M., Pitois, S., Creach, V., Simpson, S.D., Dye, S., Bear, E., Pinnegar, J.K., Harmful algal blooms and climate change: exploring future distribution changes, ICES Journal of Marine Science, Volume 75, Issue 6, November-December 2018, Pages 1882–1893, https://doi.org/10.1093/icesjms/fsy113

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona,. N & Martinez-Urtaza, J. Non-Cholera Vibrios: The Microbial Barometer of Climate Change. Trends Microbiol. (2017) Jan;25(1):76-84. doi: 10.1016/j.tim.2016.09.008.

Vezzulli, L., Colwell, R.R. & Pruzzo, C. Microb Ecol (2013) 65: 817. https://doi.org/10.1007/s00248-012-0163-2

Kaysner, C.; DePaola, A. (2004) Chapter 9: *Vibrio*. In:BAM Council (eds) Bacteriological analytical manual online. US Food and Drug Administration. Available at https://www.fda.gov/food/laboratory-methods-food/bam-vibrio (accessed June 17, 2019).

Leland, N.J. , J.F. Haney and W. Young, Into the light: A method and device for plankton separation (manuscript in preparation).

USEPA. 2003. Sampling and Analytical Procedures for GLNPO's Open Lake Water Quality Survey of the Great Lakes. U.S. Environmental Protection Agency, Great Lakes National Program Office, Chicago, IL.

Leland, N.J. and Haney, J.F. (2018) Alternative Methods for Analysis of Cyanobacterial Populations in Drinking Water Supplies: Fluorometric and Toxicological Applications Using Phycocyanin. *Journal of Water Resource and Protection*, 10, 740-761. https://doi.org/10.4236/jwarp.2018.108042

Baker, A.L. et al. 2012. Phycokey -- an image based key to Algae (PS Protista), Cyanobacteria, and other aquatic objects. University of New Hampshire Center for Freshwater Biology. http://cfb.unh.edu/phycokey/phycokey.htm 3 Oct 2019.

Cooper, S. & Dolan, C. A Guide to Common Gulf of Maine, Great Bay Coast Watch National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration (NOAA) grant #NA060AR4170109 to the NH Sea Grant College Program. NH Sea Grant publication UNHMP-M-SG-06-35 Summer 2006 Additional copies are available from: NH Sea Grant Communications Kingman Farm/University of New Hampshire Durham, NH

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Oksanen, F. J., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, B., Simpson, G.L., Solymos, P., Stevens, M.H., Szoecs, E., & Wagner, E. (2019). vegan: Community Ecology Package. R package version 2.5-6. https://CRAN.R-project.org/package=vegan

Naumova, E.N.; MacNeill, I.B. Seasonality Assessment for Biosurveillance. In: Advances in Statistical Methods for the Health Sciences. Auget JL, Balakrishnan N, Mesbah M, Molenberghs G, eds.. Birkhauser. 2007. pp437–450.

Alarcon Falconi, T.M.; Cruz, M.; Naumova, E.N. The shift in seasonality of legionellosis in the U.S. Epidemiol Infect. 2018, 146, 1824-1833.

Jones, S. H. (2000). A technical characterization of estuarine and coastal New Hampshire. PREP Reports and Publications

Piscataqua Region Estuaries Partnership, "State of Our Estuaries Report 2018" (2017). PREP Reports & Publications. 391. https://scholars.unh.edu/prep/391Normandeau Associates, Inc. Piscataqua River Ecological Studies, 1979 Monitoring Studies, Report No. 10 for Public Service Company of New Hampshire..,Bedford, N.H. 502pp

Hartwick, M., and Jones, S. H. *Vibrio parahaemolyticus* Population Dynamics Within an Estuarine Microbial Community. 2016. Poster presentation. International Symposium on Microbial Ecology, Montreal, Canada.

Oberbeckmann, S.; Wichels, A.; Wiltshire, K. H.; Gerdts, G. Occurrence of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in the German Bight over a seasonal cycle. Antonie Van Leeuwenhoek 2011, 100(2), 291-307.

Deter, J., Solen, L., Antoine, V., Jaufrey, J., Annick, D., Dominique, H.H. (2010). Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. Environmental microbiology, 12(4), 929-937.

Deepanjali, A., Kumar, H., Karunasagar, I., Karunasagar, I. Seasonal Variation in Abundance of Total and Pathogenic *Vibrio parahaemolyticus* in Oysters along the Southwest Coast of India. Applied and Environmental Microbiology. (2005) 71, 7. http://aem.asm.org/content/71/7/3575.abstract

Canesi, K. L., & Rynearson, T. A. (2016). Temporal variation of Skeletonema community composition from a long-term time series in Narragansett Bay identified using high-throughput DNA sequencing. Marine Ecology Progress Series, 556, 1-16.

Mock, T., Otillar, R.P., Strauss, J., McMullan, M., Paajanen, P., Schmutz, J., Salamov, A., Sanges, R., Toseland, A., Ward, B.J. and Allen, A.E., 2017. Evolutionary genomics of the cold-adapted diatom *Fragilariopsis cylindrus*. Nature, 541(7638), p.536.

Hernández-Becerril, D. U., Nelson, N. R., Barón-Campis, S. A., & Moreno-Gufiérrez, S. P. (2013). Morphological study of two closely related marine planktonic diatoms: *Bellerochea malleus* and *Helicotheca tamesis*. Cryptogamie, Algologie, 34(3), 245-255.

Santiago, Marilene Felipe, Maria da Glória Gonçalves Silva-Cunha, Sigrid Neumann-Leitão, Kátia Muniz Pereira da Costa, Gislayne Cristina Borges Palmeira, Porto Neto, Fernando de Figueiredo, and Fabiana Santana Nunes. "Phytoplankton dynamics in a highly eutrophic estuary in tropical Brazil." Brazilian Journal of Oceanography 58, no. 3 (2010): 189-205.

Martin, J. L.; LeGresley, M. M. ; Strain, P. M. (2001). Phytoplankton monitoring in the Western Isles region of the Bay of Fundy during 1997-98. Canadian Technical Report of Fisheries and Aquatic Sciences 2349. 4: 1-85. [details]

Hällfors, G. (2004). Checklist of Baltic Sea Phytoplankton Species (including some heterotrophic protistan groups). Baltic Sea Environment Proceedings. No. 95: 210 pp., available online at http://helcom.fi/Lists/Publications/BSEP95.pdf

Fuhrman, J. A., Steele, J. A., Hewson, I., Schwalbach, M. S., Brown, M. V., Green, J. L., & Brown, J. H. (2008). A latitudinal diversity gradient in planktonic marine bacteria. Proceedings of the National Academy of Sciences, 105(22), 7774-7778.

Shapiro, B. J., & Polz, M. F. (2014). Ordering microbial diversity into ecologically and genetically cohesive units. Trends in microbiology, 22(5), 235-247.

Nilsson, W. B., Paranjpye, R. N., Hamel, O. S., Hard, C., & Strom, M. S. (2019). Vibrio parahaemolyticus risk assessment in the Pacific Northwest: it's not what's in the water. FEMS microbiology ecology, 95(4), fiz027.

Rehnstam-Holm, A. S., Godhe, A., Härnström, K., Raghunath, P., Saravanan, V., Collin, B., ... & Karunasagar, I. (2010). Association between phytoplankton and *Vibrio* spp. along the southwest coast of India: a mesocosm experiment. Aquatic Microbial Ecology, 58(2), 127-139

Venkateswaran, K., Kiiyukia, C., Nakanishi, K., Nakano, H., Matsuda, O., & Hashimoto, H. (1990). The role of sinking particles in the overwintering process of *Vibrio parahaemolyticus* in a marine environment. FEMS Microbiology Ecology, 6(2), 159-166.

Espejo, R. T., García, K., & Plaza, N. (2017). Insight into the origin and evolution of the *Vibrio parahaemolyticus* pandemic strain. Frontiers in microbiology, 8, 1397.

Turner, J. W., Berthiaume, C. T., Morales, R., Armbrust, E. V., & Strom, M. S. (2016). Genomic evidence of adaptive evolution in emergent *Vibrio parahaemolyticus* ecotypes. Elem Sci Anth, 4.

Blackwell, K. D.; Oliver, J. D. The ecology of *Vibrio vulnificus, Vibrio cholerae,* and *Vibrio parahaemolyticus* in North Carolina estuaries. The Journal of Microbiology 2008, 46(2), 146-153.

Cloern, J. E. (1996). Phytoplankton bloom dynamics in coastal ecosystems: a review with some general lessons from sustained investigation of San Francisco Bay, California. Reviews of Geophysics, 34(2), 127-168.

Trombetta, T., Vidussi, F., Mas, S., Parin, D., Simier, M., & Mostajir, B. (2019). Water temperature drives phytoplankton blooms in coastal waters. PloS one, 14(4), e0214933.

Mühlenbruch, M., Grossart, H. P., Eigemann, F., & Voss, M. (2018). Mini-review: Phytoplankton-derived polysaccharides in the marine environment and their interactions with heterotrophic bacteria. Environmental microbiology, 20(8), 2671-2685.

Güde, H. (1985). Influence of phagotrophic processes on the regeneration of nutrients in two-stage continuous culture systems. Microbial ecology, 11(3), 193-204.

Froelich, B. A.; Noble, R. T. Vibrio bacteria in raw oysters: managing risks to human health. Philosophical Transactions of the Royal Society B: Biological Sciences 2016, 371(1689), 20150209.

Figure 15 (S1). *V. parahaemolyticus,* plankton species and environmental variables by time with peak timing and confidence intervals.

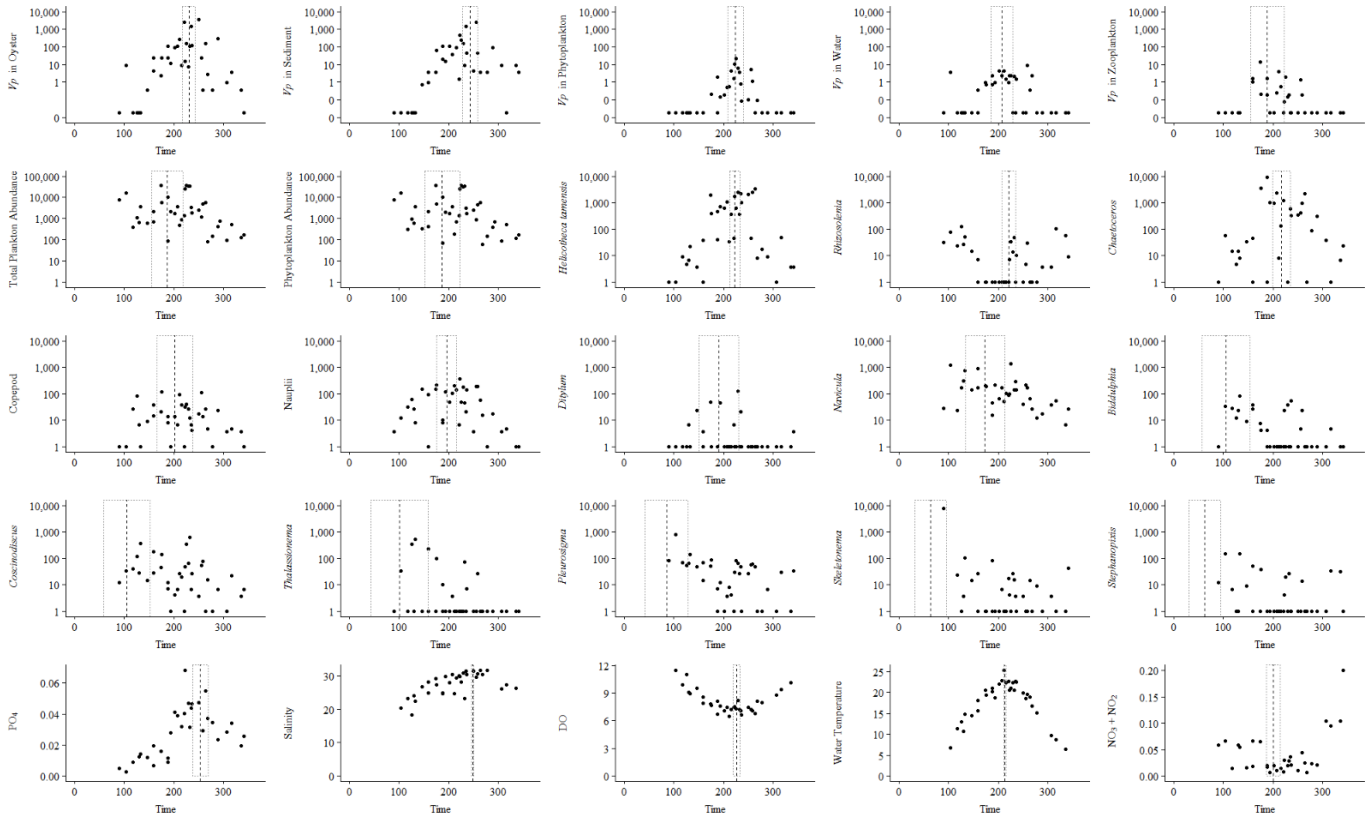


Table 15 (S2). Environmental variables showing little evidence of seasonality. Model 1 (top) and Model 2 (bottom)

	Coefficient		Standard Error		r^2	Deviance Explained	AIC	Peak Timing
	trend	seasonality	trend	seasonality				
Bacillaria	-0.001*	0.02	<0.001	0.13	0.08	0.13	129.26	
	-0.002*	0.47 0.19	<0.001	0.36 0.44	0.1	0.18	129.36	70 ± 88
Cylindrotheca	-0.002	0.05	0.001	0.17	0.02	0.07	146.57	
	-0.001	-0.45 -0.45	0.001	0.46 0.56	0.02	0.1	147.31	228 ± 78
Licmophora	-0.001	0.02	0.001	0.17	0.01	0.04	149.67	
	-0.002	0.52 0.26	0.001	0.48 0.59	0.01	0.08	150.39	64 ± 102
Thalassiosira	<0.001	-0.15	0.001	0.13	0.02	0.07	128.04	
	<0.001	0.34 0.70	0.001	0.36 0.44	0.02	0.11	128.55	26 ± 47
Tintinnida	<0.001	0.45	0.001	0.2	0.08	0.13	160.55	
	<0.001	0.57 -1.21	0.001	0.57 0.70	0.067	0.14	161.95	157 ± 56
DON	<0.001**	<0.01	<0.001	<0.001	0.26	0.31	104.87	

	<0.001***	-0.008; -0.03*	<0.001	0.01 0.01	0.27	0.34	102.12	200 ± 45
NPOC	-0.001	-0.02	<0.001	0.07	0.03	0.10	73.3	
	-0.001	0.32 0.23	<0.001	0.20 0.23	0.09	0.18	72.24	55 ± 56
TDN	<0.001	-0.01	<0.001	<0.001	0.11	0.17	82.39	
	<0.001	<0.001 <0.001**	<0.001	<0.001	0.09	0.18	81.07	189 ± 41
NH4	<0.001	<0.001*	<0.001	<0.001	0.20	0.24	156.49	
	<0.001*	<0.001 <0.001	<0.001	<0.001	0.16	0.24	15.82	158 ± 62
TSS	0.008	2.46	0.01	1.51	0.03	0.10	253.48	
	0.005	9.22 -3.31	0.01	4.22 4.89	0.12	0.21	251.54	111 ± 60
PC	-0.0003	0.08	0.0005	0.07	0.006	0.06	71.61	
	-0.0005	0.44 -0.02	0.0005	0.20 0.24	0.09	0.19	69.19	94 ± 61
PN	<0.001	0.02	<0.001	0.001	0.02	0.08	40.66	
	<0.001	<0.001 <0.001	<0.001	<0.001	0.02	0.13	40.08	126 ± 86
DON	<0.001***	<0.001	<0.001	<0.001	0.20	0.31	104.87	
	<0.001	<0.001 <0.001	<0.001	<0.001	0.26	0.33	103.93	200 ± 46
CHL	-0.001	0.08	0.003	0.45	0.06	0.01	193.56	
	-0.002	1.44 0.48	0.003	1.37 1.53	0.06	0.04	194.43	72 ± 107
Pheophytin	<0.001	-0.02	0.001	0.22	0.06	0.01	146.72	
	<0.001	0.06 0.11	0.002	0.67 0.75	0.09	0.01	148.7	31 ± 200
pH	<0.001	-0.01	<0.001	0.01	0.05	0.11	46.66	
	<0.001	<0.001 <0.001	<0.001	<0.001	0.10	0.19	47.64	30 ± 36
Turbidity	<0.001	-0.93	0.006	1.03	0.04	0.03	247.21	
	0.001	-2.27 1.39	0.006	2.80 3.70	0.06	0.04	248.83	126 ± 166

significance of coefficients indicated as *** 0.001, ** 0.01, * 0.1

Chapter 3

Adaptations of an Environmental Population of *Vibrio parahaemolyticus* in the Great Bay Estuary

Meghan Hartwick[a,b] , Cheryl Whistler [a,b], Vaughn Cooper [a,d], Stephen Jones [a,c]
[a]Northeast Center for Vibrio Disease and Ecology, University of New Hampshire, Durham, NH; mah2002@wildcats.unh.edu
[b]Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH; Cheryl.Whistler@unh.edu
[c]Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH; Stephen.Jones@unh.edu
[d]Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA; vaughn.cooper@pitt.edu

ABSTRACT

Vibrio parahaemolyticus is a leading cause of foodborne illness, and the intrinsic link it has with the environment it inhabits has been well studied and characterized. A wide range of environmental conditions and ecological interactions has been reported to influence, or at least correlate with *V. parahaemolyticus* concentrations. The complexity of this ecology coupled with the notable differences observed in drivers of *V. parahaemolyticus* dynamics has led to the observation that a combination of environmental factors including fine-scale differentiation of the *V. parahaemolyticus* population into genetically distinct sub populations may contribute conditions that differentially promote human health risk. *V. parahaemolyticus* isolates collected over the course of one year from oysters, phytoplankton and zooplankton at two sites in the Great Bay estuary (GBE) were sequenced with Illumina Hi-Seq and Oxford Nanopore Technology to assess *V. parahaemolyticus* for evidence of local adaptation or ecotype differentiation between isolates by site, matrix and season. Forty-one unique sequence types (ST) were identified in the population and sixteen of these were previously reported on the pubMLST *V. parahaemolyticus* ST database. Though lineage most strongly predicted genetic diversity between isolates in the GBE, evidence of horizontal exchange of plasmids and niche specific-genetic content was observed, and this combination of genetic content through lineage and lifestyle masked potential ecotype differentiation. The recently proposed *V. parahaemolyticus* cgMLST and isolates in ST 12, 49, 57 and 114 collected from locations outside the GBE were also compared to determine if an ecological signal could be identified in evolutionary patterns.

1. INTRODUCTION

Vibrio parahaemolyticus is a leading cause of foodborne illness and is naturally occurring in the environment where it persists through nutrient cycling and contributes to the food web. Whereas most strains are believed to be non-pathogenic, a subset of the species can cause gastroenteritis and septicemia in humans (FDA, 2005). Strains that cause human disease are most often contracted from the consumption of raw or undercooked shellfish and other seafood in warm water regions and more recently in temperate regions (FDA, 2005; McLaughlin et al., 2005). The emergence and establishment of *V. parahaemolyticus* as a causative agent of shellfish-borne illnesses in the Northeast United States has stimulated keen interest in understanding the environmental conditions that may influence human health risk in this region.

The ecology of *Vibrio parahaemolyticus* has been well studied and characterized (eg: Lovell, 2017; Davis et al., 2017; Urquhart et al. 2016; Lopez-Hernandez et al., 2015; Turner et al., 2014;Martinez-Urtaza et al. 2012; Oberbeckman et al., 2011; Vezulli et al., 2009; Baker-Austin et al. 2010, Deepanjali et al., 2005, Jones and Summer-Brason 1998, DePaola et al., 1990, Colwell et al., 1973). Whether shellfish are a preferred environment for *V. parahaemolyticus* remains unclear, however because of their commercial importance, the shellfish-*V. parahaemolyticus* relationship is the most frequently studied interaction (Lovell et al., 2017; DePaola et al. 1990, 2003; Zimmerman et al. 2007). Filter feeding of suspended material is the most likely route by which *V. parahaemolyticus* becomes concentrated in shellfish (e.g., Froelich et al. 2013). Given this, *V. parahaemolyticus* associated with copepods and chitinous phytoplankton are frequently studied as they may relate to *V. parahaemolyticus* dynamics in shellfish (Caburlotto et al., 2010; Takemura et al., 2014, Turner, 2009 and 2014, Paranjpye et al., 2015, Gilbert et al., 2012, Grimes et al., 2014 Martinez-Urtaza et al., 2012, Asplunde et al. 2011, Beveridge et al., 2010, Kaneko and Colwell, 1975).

Concentrations of *Vibrio parahaemolyticus* in the Great Bay estuary (GBE) from different environmental matrices have distinctly different seasonal patterns and peak timing, especially between *V. parahaemolyticus* in oysters, phyto and zooplankton (Hartwick et al., in prep) suggesting that the dynamics of *V. parahaemolyticus* may vary by niche in the environment. These differences in *V. parahaemolyticus* dynamics between matrices have been observed elsewhere in

both its variable detection rate as well as the environmental conditions that *V. parahaemolyticus* is associated with (Takemura et al., 2014). This type of ecological differentiation has been studied as it may relate to underlying genetic differences in *V. parahaemolyticus*. Johnson et al., (2012) and Shapiro and Polz (2014) reported *V. parahaemolyticus* strains from similar environmental niches were more similar in genetic content and allelic diversity than they were to strains that were isolated from different environmental conditions. Genetically distinct sub-populations of *V. parahaemolyticus* constrained by geographic barriers have also been observed (Yang et al., 2019; Cui et al., 2015). Further, Keymer et al., (2007) and Hunt et al., (2008) identified potential differentiation in other *Vibrio* spp. by allelic variation or variable genome content that was linked to the distinct ecological-niche from which strains were isolated. These observations are part of the basis for proposing that *Vibrio parahaemolyticus* populations may be structured into ecological species, or ecotypes.

Ecotypes can be understood as sub-populations that interact with other strains within their niche but have limited exposure to *V. parahaemolyticus* that do not interact within the same microhabitats in the environment. Because of this genetic isolation and the niche-specific conditions, strains that inhabit similar niches will have more similar allelic diversity and accessary genomes (Freidman, Alm and Shapiro, 2012). *V. parahaemolyticus* is an ideal candidate to investigate the potential of differentiation by micro-environment within a population. The genome is generally composed of two circular chromosomes with ~5,000 genes (Makino et al., 2003), approximately half of which are conserved, and the remaining genome can be highly variable even between closely related strains (Gonzalez-Escalono et al., 2016). This variable content can be acquired through multiple routes including horizontal gene transfer mediated by conjugation between individuals, transduction via phage and chitin-induced competence (Le Roux and Blokesch, 2018). Allelic diversity of the material already present in the genome can be introduced via homologous recombination and mutation (Bobay and Ochman, 2017, Ceccarelli et al., 2013; Johnson, 2013). These mechanisms contribute to the standing genetic diversity of housekeeping genes, core genes and variable genome content and may result in the differentiation of sub populations of *V. parahaemolyticus* into ecotypes if the rate of gene flow is variable within environments compared to between environments (Shapiro and Polz, 2014,

Cordero and Polz, 2014, Hunt et al., 2008, Johnson et al., 2006; Cohan et al., 2001). This can in turn provide information about the ecological drivers that are shaping the population based on prevalence of that shared characteristic to contribute to persistence within the local population (Metzger and Blokesch, 2014).

Given the high amount of genetic diversity of *Vibrio parahaemolyticus* within the GBE (Ellis et al., 2012) and the variability of seasonal dynamics of *V. parahaemolyticus* (Hartwick et al., 2019; in prep.), isolates from oysters, phyto and zooplankton collected throughout 2014 were assembled using a combination of Illumina and Oxford Nanopore Technology (ONT) for whole genome analysis to determine how the observed environmental variability could be better understood in light of patterns of underlying genetic population diversity. In addition to their potential contribution to the accumulation of *V. parahaemolyticus* in oysters, *V. parahaemolyticus* isolates from phyto and zooplankton were of interest to determine if differences in diversity could be detected from isolates directly cultured from chitin-rich niches based on the potential of chitin-induced competence to facilitate horizontal gene transfer.

An important yet under studied challenge for developing broadly useful methods for monitoring and forecasting *V. parahaemolyticus* risk is the influence of unique sets of environmental variables on the location and population specific genetic and genomic adaptations that lead to *V. parahaemolyticus* population variations at regional and even the harvest area scales (Noble and Froelich, 2016). Therefore, providing context to the relationship between genetic diversity and the environment is an important direction to better understand how the environment shapes *V. parahaemolyticus* dynamics in order to identify the conditions that are associated with human-health risk. The potential for location-specific adaptations of *V. parahaemolyticus* to the ecology of the GBE observed in this study is an important step to connect environmental conditions to population dynamics and provides new targets for identifying the environmental variables that influence *V. parahaemolyticus* concentrations in oysters.

2. METHODS

2.1. Study sites, environmental sampling and bacterial analysis

The study area was focused on natural oyster beds found in the Great Bay estuary (GBE) of New Hampshire and Maine. Nannie Island (NI) in Great Bay is next to a significant oyster (*Crassostrea virginica*) bed and the other oyster bed site was in the Oyster River (OR) (Fig. 16), both being long-term Vibrio monitoring locations (Urquhart et al. 2015; Jones and Summer-Brason 1998). The GBE has been monitored for over 30 consecutive years (March-December) by multiple agencies, including the Great Bay National Estuarine Research Reserve (GBNERR) and their System Wide Monitoring Program (SWMP; https://cdmo.baruch.sc.edu/dges/). Continuous (Q15) temperature, salinity, dissolved oxygen (DO), pH, and turbidity data were obtained from SWMP from 2014-2016 for times simultaneous with and preceding sampling. Nutrients at the time of collection (NPOC, TDN, NO3.NO2, NH4, PO4, DON, TSS, PC, PN, Chlorophyll *a* and Pheophytin measurements) were obtained from SWMP and from grab samples collected in 1L acid washed Nalgene Bottles analyzed by the University of New Hampshire Water Quality Analysis Lab for the NI study site.

Fig. 16. The GBE Study area on the border of Maine and New Hampshire**.** OR = Oyster River; NI = Nannie Island

Oyster and plankton samples were collected concurrently with water temperature, salinity, pH and DO using YSI 6600 and EXO multiprobe sondes (Yellow Springs Instruments, Yellow Springs, Ohio), during low tide from the NI and OR study sites. Plankton samples were collected using a 53 μm mesh net (Aquatic Instruments). Oyster, phyto- and zooplankton samples were processed and analyzed for *V. parahaemolyticus* detection according to previously published methods (Hartwick et al., 2019) with alkaline peptone water enrichment, Vibrio CHROMAgar and the FDA established PCR and MPN calculations at UNH Jackson Estuarine Lab (JEL). Probable *Vibrio* isolates were confirmed as *V. parahaemolyticus* by PCR detection of the *tlh* gene (Urquhart et al., 2016, Hartwick et al., 2019, Kaysner and DePaola, 2004).

2.2 Illumina sequencing

2.2.1 Isolate selection and Ssquencing

All isolates from 2014 oyster, phytoplankton and zooplankton were considered for genomic analysis. Isolates from were cultured overnight in HI broth and genomic DNA from each isolate was isolated with the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). DNA quality and concentrations were evaluated with NanoDrop 1000 (Thermo Scientific, Rockford, IL). The final concentration of high-quality extractions was quantified using a Qubit double-stranded DNA HS assay kit and a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY). Libraries for Illumina sequencing were generated using the TruSeq Kit (Illumina). One hundred and five unique isolates were submitted to the UNH Hubbard Genome Center for Illumina-Hi Seq 250bp paired-end sequencing.

2.2.2 Assembly and annotation

Sequenced forward and reverse reads were quality checked with FASTQC (Page, 2010). Adapter and low-quality reads were trimmed with Trimmomatic (Bolger et al., 2014) then assembled with SPAdes (Nurk et al., 2013). Assembly quality was assessed with Quast (Gurevich et al., 2013). A custom python script was used to remove contigs with <10x coverage. Twenty isolates were not

included in further analysis based on low sequencing quality or poor assembly quality in the QC step. Eighty-five assembled genomes were annotated with Prokka (Seemann, 2014) using the genus setting and the *Vibrio* database. Sequence Type (ST) was assigned by SRST2 (Inouye et al., 2014) based on the following genes: *dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC* and *tnaA* from the *V. parahaemolyticus* MLST (http://pubmlst.org/*V. parahaemolyticus)*. Previously unidentified ST were identified as ST a through ST f. The sequences for all 85 *V. parahaemolyticus* strains are available in SRA under the accession numbers listed below (Table 16).

Table 16. Sample ID, Date of collection, Site, Sample source and SRA Accession # for 85 *V. parahaemolyticus* isolates from 2014.

Isolate ID	Date	Site	Sample Source	Accession #
4974	6/20/2014	NI	OYS	
4973	6/20/2014	NI	OYS	
5129	7/7/2014	NI	OYS	
5132	7/7/2014	NI	OYS	
5135	7/7/2014	NI	OYS	
5152	7/7/2014	NI	OYS	
5134	7/7/2014	NI	OYS	
5196	7/7/2014	NI	OYS	
5167	7/7/2014	NI	PHYTO	
5171	7/7/2014	NI	PHYTO	
5170	7/7/2014	NI	PHYTO	
5166	7/7/2014	NI	PHYTO	
5168	7/7/2014	NI	PHYTO	
5159	7/7/2014	NI	ZOO	
5155	7/7/2014	NI	ZOO	
5157	7/7/2014	NI	ZOO	
5162	7/7/2014	NI	ZOO	
5163	7/7/2014	NI	ZOO	
5165	7/7/2014	NI	ZOO	
5707	8/13/2014	NI	OYS	
5844	8/20/2014	NI	OYS	
5848	8/20/2014	NI	OYS	
5886	8/20/2014	NI	PHYTO	
5899	8/20/2014	NI	ZOO	
6217	9/25/2014	NI	OYS	
6213	9/25/2014	NI	OYS	
6216	9/25/2014	NI	OYS	
6218	9/25/2014	NI	OYS	
6233	9/25/2014	NI	PHYTO	
6235	9/25/2014	NI	PHYTO	
6237	9/25/2014	NI	PHYTO	
6354	10/28/2014	NI	OYS	
6362	11/13/2014	NI	PHYTO	
6379	12/1/2014	NI	OYS	
6380	12/1/2014	NI	OYS	
6382	12/1/2014	NI	OYS	

5055	7/7/2014	OR	OYS
5053	7/7/2014	OR	OYS
5047	7/7/2014	OR	OYS
5049	7/7/2014	OR	OYS
5068	7/7/2014	OR	OYS
5057	7/7/2014	OR	OYS
5051	7/7/2014	OR	OYS
5061	7/7/2014	OR	OYS
5066	7/7/2014	OR	OYS
5111	7/7/2014	OR	PHYTO
5117	7/7/2014	OR	PHYTO
5122	7/7/2014	OR	PHYTO
5125	7/7/2014	OR	PHYTO
5115	7/7/2014	OR	PHYTO
5119	7/7/2014	OR	PHYTO
5107	7/7/2014	OR	ZOO
5103	7/7/2014	OR	ZOO
5106	7/7/2014	OR	ZOO
5109	7/7/2014	OR	ZOO
5101	7/7/2014	OR	ZOO
5105	7/7/2014	OR	ZOO
5858	8/20/2014	OR	OYS
5864	8/20/2014	OR	OYS
5870	8/20/2014	OR	OYS
5902	8/20/2014	OR	ZOO
6241	9/25/2014	OR	OYS
6247	9/25/2014	OR	OYS
6248	9/25/2014	OR	OYS
6250	9/25/2014	OR	OYS
6249	9/25/2014	OR	OYS
6246	9/25/2014	OR	OYS
6242	9/25/2014	OR	OYS
6243	9/25/2014	OR	OYS
6244	9/25/2014	OR	OYS
6224	9/25/2014	OR	PHYTO
6219	9/25/2014	OR	PHYTO
6226	9/25/2014	OR	PHYTO
6221	9/25/2014	OR	PHYTO
6232	9/25/2014	OR	ZOO
6231	9/25/2014	OR	ZOO
6229	9/25/2014	OR	ZOO
6228	9/25/2014	OR	ZOO
6366	11/13/2014	OR	OYS
6364	11/13/2014	OR	OYS
6365	11/13/2014	OR	OYS
6367	11/13/2014	OR	OYS
6363	11/13/2014	OR	PHYTO
6376	12/1/2014	OR	OYS
6377	12/1/2014	OR	OYS

2.3 Gene content and pangenome analysis

Pangenome analysis was conducted with Roary (Page et al., 2015), BLASTP identity was set to 90% and consideration of genes to be core (or variable) was based on the presence of the gene in at least 98% percent of isolates (~83/85). Gene Ontology (GO) was assigned using Blast+ (Altschul et al., 1990) and the Uniref90 and UniprotKB databases (The UniProt Consortium, 2019). The highest identity match from BLASTX of was selected based on 100 results. Gene function was assigned with GO.db (Carlson, 2019) and GO categories were assessed using the highest order GO terms within the classification of biological processes, molecular function and cellular process.

2.4 Nanopore sequencing

Previously extracted gDNA (section 2.2.1) from representative isolates of each clonal cluster were re-sequenced using the Nanopore ligation sequencing kit 1D (R9 version) for MinION sequencing. Unicycler (Wick et al., 2017) was used to produce Nanopore and Illumina hybrid assemblies and annotated with Prokka (Seemann, 2014). Assembly quality were evaluated with Quast (Gurevich et al., 2013) and visualized with Bandage (Wick et al., 2015). Ten isolates were assembled to a minimum coverage of 30x and resolved to two chromosomes with hybrid ONT and Illumina sequenced reads.

2.5 Allelic diversity

Hybrid Nanopore-Illumina sequenced and assembled isolates were aligned with Minimap (Li, 2018) and annotated variant call files (VCF) were constructed with SAMtools and BCFtools (Li et al., 2009) or a custom bash pipeline of gene alignment files from Roary (Page et al., 2015) and SNP-sites (Page et al., 2016). VCFtools (Danecek et al., 2011) was used to filter SNPS by quality > 60. Phylogenetics relationships between isolates in the study were assessed using MLST, core genes and variable genes aligned in MAFFT (Katoh & Standley, 2013) and PRANK (Löytynoja, A. 2014). Maximum likelihood trees were constructed in RAxML (Stamatakis, 2014). Relationships were visualized using the ggtree package developed for R (Yu et al., 2017).

2.6 Local adaptation

2.6.1 Chromosomal distribution of core genome content

The core genome content identified by Roary (Page et al., 2015) analysis was compared to the *V. parahaemolyticus* core genome (Gonzelez-Escalono et al., 2016). The FASTA and GBK files of *V. parahaemolyticus* isolates resolved to 2 chromosomes and categorized as 'complete' from NCBI (Coordinators, N.R., 2016) were accessed on 9/19/2019. Gene location of the core genes in the GBE *V. parahaemolyticus* population was compared with chromosomal gene location from the geographically distinct closed NCBI *V. parahaemolyticus* genomes.

2.6.2 Global ST and local adaptation

Custom scripts combining the SRA query tool (Leinonen et al., 2010) and SRST2 (Inouye et al., 2014) were used to query to the *Vibrio parahaemolyticus* short-read archive for genomes with ST identified in the eighty-five genomes included in this study. Seven isolates with matching ST were identified, assembled and annotated using the pipeline specified in section 2.2.2. Gene content and allelic diversity were analyzed as described in section 2.3 to look for evidence of genetic change in lineages that were isolated within the GBE compared to matching ST from other regions.

2.7 Genome wide association studies

Genome wide association studies (GWAS) between genomic content, function or allelic diversity and ST, date, site and matrix was performed using a combination of Scoary (Brynildsrud et al., 2016), *TopGo* (Rahnenfuhrer, 2019) and multivariate analysis with Multiple Permutation Response Procedure (MRPP), Non Metric Ordination Analysis (NMDS) with *vegan*: Community Ecology Package, 2.5-2 (Oksanen et al., 2018), and Indicator Species Analysis (ISA): *indicspecies* (De Caceres and Legendre, 2009) in R version 3.5.3 (R Core Team, 2019).

3. Results

Eighty-five isolates from oysters, phyto and zooplankton were collected from June to December in 2014 and sequenced with Illumina Hi-Seq to a minimum 10x coverage (Figure 17). The average number of base pairs per genome was 5,080,909 ± 82,643 and these resolved to an

average of 97 ±49 contigs. Slightly fewer isolates were obtained from NI (n=39) than OR (n=46) and the majority (73%, 62/85) were from sampling dates in early July (n=38) and late August (n=24). Isolates were cultured from oysters throughout the sampling season, whereas the majority of isolates from phytoplankton and zooplankton could only be cultured from July through September.

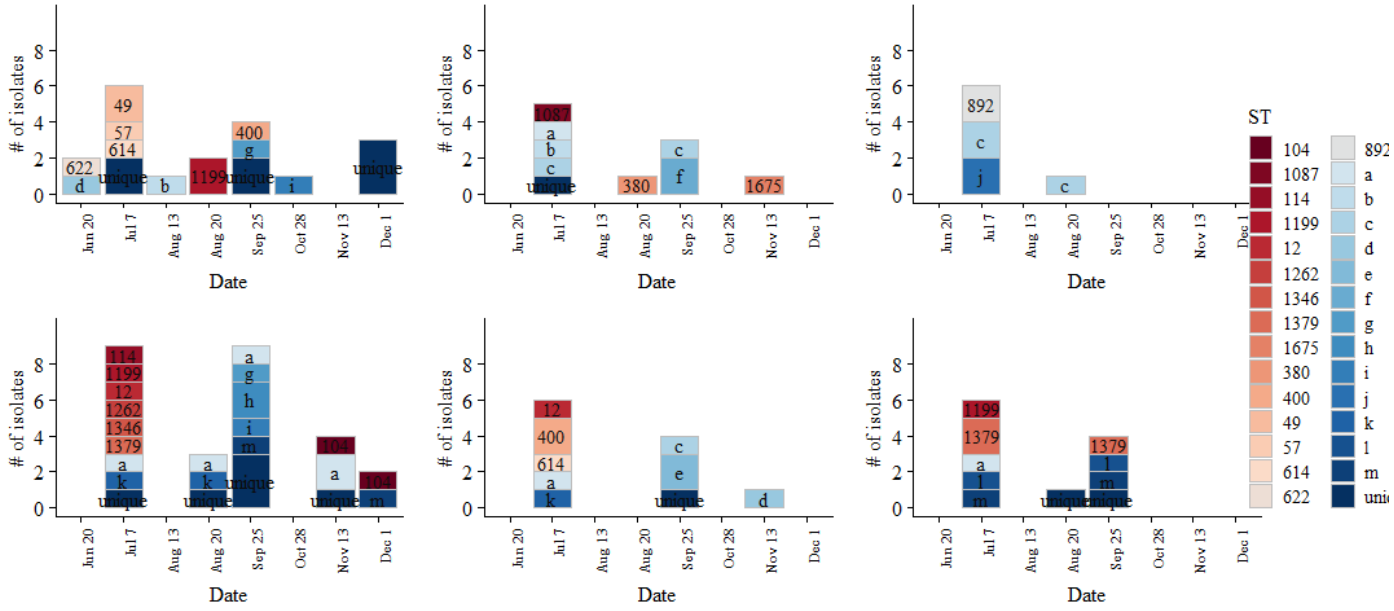


Figure 17. The distribution of *V. parahaemolyticus* sequence types during 2014 in different ecological matrices. (clockwise), isolates collected from (a) NI oyster, (b) NI phyto, (c) NI zooplankton, (d) OR oysters, (e) OR phytoplankton and (f) OR zooplankton between June and December 2014. Previously identified sequence types are in red and previously unidentified sequence types seen more than once are blue. Unidentified sequence types seen only once are in dark blue and identified as 'unique'.

3.1. Overall diversity

3.1.1 Sequence type diversity

The diversity of the isolates detected in this study was high. Forty-six unique ST were identified by date, site and by sample source (Figure 2). Some evidence of clonality was observed. For example, twenty-one ST were collected at least twice, and these were approximately split between previously identified ST (n=8) and novel ST (n=13). Though clonal STs were occasionally detected from the same culture scheme (n = 7), the majority of isolates with clonal ST were detected from disparate sampling events, sites and sample sources. For example, ST 49 was detected only once during the study from NI oysters on July 7th, whereas ST 1199 was detected

from OR oysters and zooplankton on July 7th and was later isolated from NI oysters on August 20th. Two previously unidentified sequence types (ST a and c) were detected frequently throughout the study from both sites and most matrices. Twenty-five ST were detected only once during the study and were predominantly previously unreported STs (n=17).

3.1.2 Content and function in the pangenome

The individual gene content of each isolate was approximately 4557 ± 90 genes (minimum = 4390, maximum = 4822 CDS), for a total of 387,458 genes combined between the 85 isolates. This was resolved to 13,847 unique genes in the pangenome including two plasmids (Figure 17). 3,931 genes were shared (core) by at least 98% of isolates (83 of 85), 7,133 genes were present in 2 to 82 isolates and 3,284 genes only occurred once (accessory). Overall, the majority (65% = 9,000/13,847) of the genes were identified as hypothetical proteins and 35% (4,846/13,847) had assigned gene identifiers and functional annotation. The majority of genes in the core genome were previously identified with assigned gene ids and annotation (71.8%= 2609/3755) and the genes in the accessory genome were predominantly hypothetical proteins (75%, 7917/10,417).

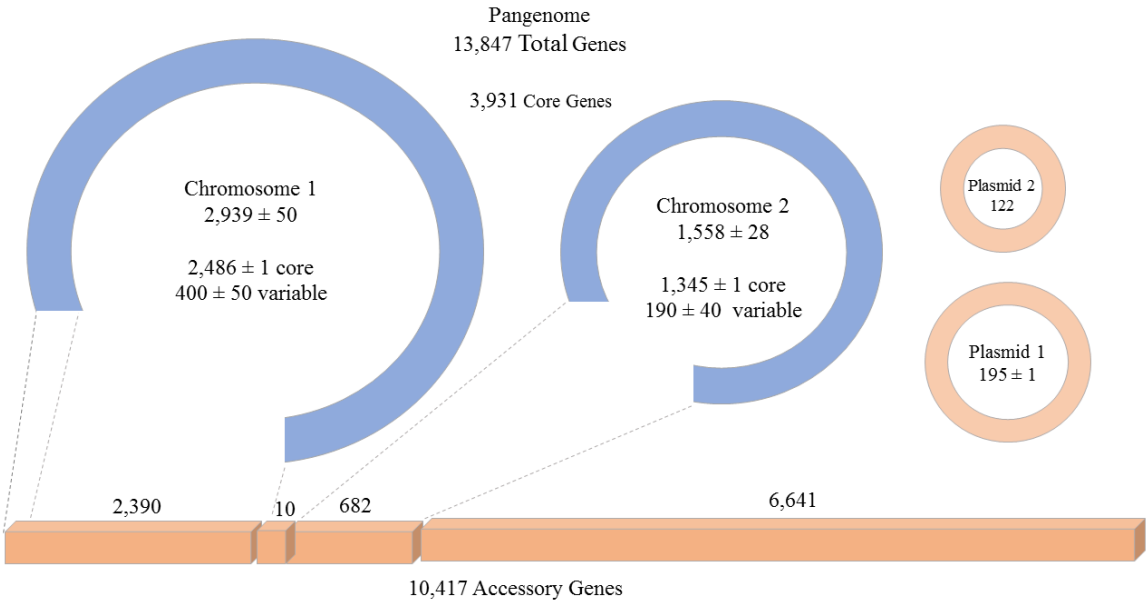


Figure 18. The pangenome of 85 *V. parahaemolyticus* isolates from the GBE. The core genome is blue and accessory content is orange.

In total, 7,206 of the 13,847 genes in the pangenome were mapped to chromosomes or plasmids, and 6,641 genes in the accessory genome were not present in the ten Nanopore-Illumina hybrid assembled genomes and so their location was not determined (Table 17, Figure 18). Chromosome one contained on average 2,939 ± 50 coding sequences and chromosome two had approximately 1,558 ± 28 coding sequences. Genes in the core and accessory genome were present on both the first and second chromosome. The first chromosome was composed of approximately 2,486 ± 1 core genes with matching identifiers and approximately 400 ± 50 genes that were variable. The second chromosome contained approximately 1,345 ± 1 of the genes from the core genome and 190 ± 40 variable genes. Twelve genes in the accessory genome mapped to the first chromosome in isolates 5171, 5055 and 6365 whereas in isolate 5057 they were mapped to chromosome two. The annotated genes were identified as Modulator of *FtsH* protease *HflC*, Modulator of *FtsH* protease *HflK*, Cold shock-like protein *CspG*, Na(+)/H(+) antiporter *NhaA* and seven were identified as hypothetical proteins. These ten genes were also present in 34 ± 3 isolates. The core genes found on chromosome one and two were similarly split between previously annotated genes and those annotated as hypothetical proteins (annotated on chromosome 1 = 1888/2486, chromosome 2 = 883/1346).

Table 17. The genes of ten unique ST resolved to two chromosomes and plasmids**.**

ID	Coverage	# of Contigs	ST	Chromosome 1			Chromosome 2			Plasmid		
				Total	Core	Variable	Total	Core	Variable	Total	Core	Variable
5055	102.2	3	12	3066	2484	583	1555	1346	209	195	0	195
5057	86.7	3	1379	2939	2486	453	1601	1346	255	194	0	194
5061	157.3	2	K	2899	2486	413	1552	1345	207	-	-	-
5165	33.8	2	J	2932	2486	446	1584	1345	239	-	-	-
5171	145.8	3	A	2933	2486	447	1544	1346	198	122	0	122
6213	26. 1	2	G	2888	2486	402	1599	1344	255	-	-	-
6233	135.2	2	C	2919	2486	430	1527	1346	181	-	-	-
6248	37	2	H	2970	2486	484	1517	1346	171	-	-	-
6249	65.6	2	I	2915	2485	430	1559	1346	213	-	-	-
6365	56.4	3	A	2933	2486	447	1544	1346	122	122	-	122

Two plasmids with unique gene content were identified in the Nanopore-Illumina hybrid assembled genomes. The larger plasmid, that was present in isolates 5055 and 5057 (Table 17),

was also part of the genome of isolates 5101, 5103 and 5155. The smaller plasmid, that was present in 5171 and 6365, was also in the genomes of isolates 5051, 5109, 5115, 5858, 6241 and 6364. In the larger plasmid, 21/194 genes had gene ids and functional annotation whereas the majority (89%, 173/194) of genes were annotated as hypothetical proteins. Similarly, there were 13/122 genes in the smaller plasmid with gene ids and annotation, whereas the majority (89%, 109/122) of genes were annotated as hypothetical proteins.

9,510 genes in the pangenome were annotated in the uniprotKB database and 5100 of these also had functional gene ontology (GO) for either molecular (n= 3742), cellular (n= 2377) or biological function (n=2050). Only 741 genes were annotated for all function GO groups. The majority of genes annotated in the cellular component group were related to cellular structure function and maintenance of cell membrane (GO:0016021, n=1288), cytosol (GO:0005737, n = 370) and ATP-binding (GO:0043190, n = 36). Multiple genes were also related to bacterial flagellum development (GO:0009288, n = 25), type II (GO:0015627, n = 3) and type III secretion systems (GO:0030257, n = 3). Approximately one third (1279 genes) annotated with molecular function GO were involved in binding (GO = GO:0005488). DNA binding (GO:0003677, n=549), ATP binding (GO:0005524, n= 201) and RNA binding (GO:0003723, n = 47) represented the majority of genes related to this function, but various metal ion binding functions were also present. The other major group within molecular function involved genes related to catalytic activity. The majority of genes in this group were involved with transferase activity (GO:0016740, n=281), hydrolase activity (GO:0016787, n = 139), and sixteen genes were involved with recombinase activity (GO:0000150).

Genes annotated to biological process (BP) were of particular interest in this study as they contain terms that relate most closely with environmental interactions. The initial overall query of the BP function in the pangenome suggested that cellular processes (GO:0009987, GO:0008151, GO:0044763, GO:0050875) represented the largest group of GO annotated genes (n=1,501). However, further investigation showed that this was mostly related to redundancies in the acyclic GO graphs. The majority of genes within cellular process were annotated to metabolism (n=1,369), as well as response to stimulus (n = 194). This was confirmed by individual queries of

metabolic processes (GO:0008152, GO:0044236, GO:0044710) and response to stimuli (GO:0050896, GO:0051869). Within metabolic processes GO terms, 805 genes were related to cellular nitrogen metabolic processes (GO:0034641), 180 genes were associated with carbohydrate metabolic processes and 105 genes were annotated to cellular phosphorous metabolic processes (GO:0006793). Genes related to response to stimulus were attributed to stress response (n= 71) including; heat (n = 7), cold (n= 2), starvation (n= 1) and phage response (n= 1). In addition, genes related to localization (n= 305), locomotion (n =71), adhesion (n=15) and cell killing (n= 1) were also identified.

The genes with GO BP annotation in the core (n=1,335) were found on both the first (n= 1,000) and second chromosome (n = 335). Overall the gene function in the core genome at the highest level of the acyclic GO hierarchical structuring was significantly enriched in functions related to metabolic processes including: organonitrogen compound metabolic processes, organic acid metabolic process, oxoacid metabolic process, carboxylic acid metabolic process, small molecule metabolic process, phosphate-containing compound metabolic processes and protein metabolic process ($p < 0.001$). At more specific resolution, significant enrichment was present in the core genome in functions related to gene expression, RNA processing and modification, cell cycle and translation ($p > 0.001$). Differences were observed between the distribution of function in the core genome between the first and second chromosome (Figure 18). For example, relatively more genes involved with metabolism and biogenesis were on the first chromosome whereas the majority of genes involved with stress response and flagellar locomotion or taxis were found on the second chromosome. At the highest level of the acyclic hierarchical structure of the GO structure, cellular metabolic process, primary metabolic process, nitrogen compound metabolic process, organic substance metabolic process were significantly enriched in the core genes on the first chromosome, whereas localization, biological regulation, signaling and transport were significantly enriched in the core content on the second chromosome including response to stimulus and flagellar development and regulation ($p < 0.001$).

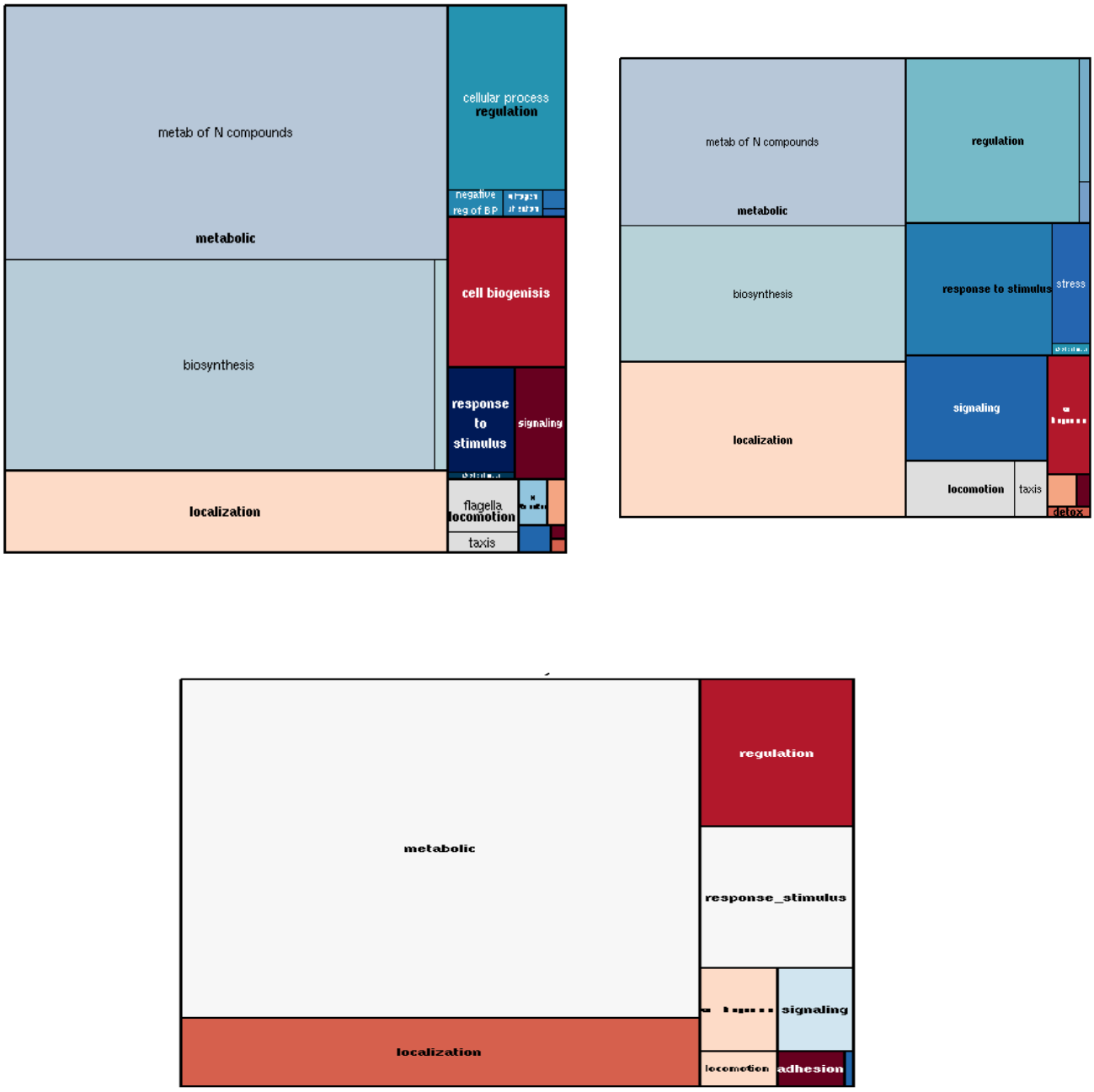


Figure. 19. GO annotated function in the core genome on chromosome one and two and the variable genes in the accessory genome of *V. parahaemolyticus* isolates from the Great Bay estuary.

The BP annotated variable genes in the accessory genome (n=714) were enriched for function related to DNA metabolic process, specifically nucleic acid metabolic process, DNA recombination, DNA modification, DNA integration and transposition ($p > 0.001$). Within more specific functions in the acyclic structure, variable genome content was enriched for DNA

alkylation, DNA methylation, defense response, clearance of foreign intracellular DNA, viral process, life cycle, and virion assembly, symbiont process, multi-organism process, interspecies interaction, O antigen biosynthetic process, O antigen metabolic process, response to stress and cell adhesion ($p > 0.001$) (Figure 18). The variable content that was annotated with BP GO function was mapped to the first or second chromosome (n=294) or were from isolates where their location was not resolved (n=420). Both plasmids contained fewer than 10 BP GO annotated genes. Genes for sporulation inhabitation, and chromosome partitioning were found on both. The larger plasmid was annotated with genes related to DNA modification whereas the smaller plasmid was annotated with two genes for antitoxin (*chpS*, *higA-1*) and a cold shock protein (*cspG*).

3.2 Local adaptation

The potential ecological relevance of the diversity of the isolates from the GBE was also assessed in relationship to *Vibrio parahaemolyticus* collected from outside the GBE. The core genome in the GBE (3,931 genes) consisted of approximately 1,677 more genes than the 2,254 genes cgMLST suggested by Gonzalez-Escalona et al., (2017). It is important to note that only 64 genes from the cgMLST were not present in in the GBE core genome and the majority of these (52/64) were annotated as hypothetical proteins. The core content that was part of the cgMLST was mapped to either the first (n= 1,563) or second chromosome (n=624) chromosome. Likewise, the additional 1,677 genes in the GBE core genome were found on either the first (n= 923) or second (n=722) chromosome (Figure 20). The *V. parahaemolyticus* cgMLST was significantly enriched for genes involved with metabolism, specifically, organonitrogen compound metabolic process and protein metabolic processes and translation and cell cycle processes ($p > 0.001$), whereas the genes in the extra GBE core were enriched in functions related to localization, cell projection organization, cellular response to stimulus, transport and signaling ($p > 0.05$). Further work is needed to assess the biological validity of these differences based on the relatively low number of genes assigned with GO terms. For example, if there were significantly more annotated genes in the extra GBE core that mapped to the second chromosome and likewise more annotated genes in the cgMLST core that mapped to the first chromosome, then that could account for the differences in the functional enrichment between the cgMLST and the extra GBE core.

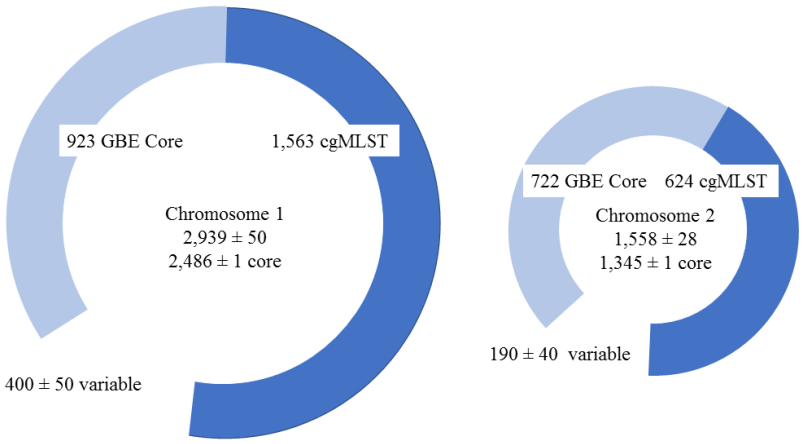


Figure. 20. The core and variable genome of the pangenome of isolates in this collection (light blue) compared to genes in the *V. parahaemolyticus* cgMLST (dark blue) on chromosomes one and two.

The potential ecological importance of the accessory genome from the GBE was assessed by comparing GBE isolates to isolates of the same ST isolated from other locations. Thirty records for ST 12, 49, 57, 104, 114, 380, 400, 614, 892, 1087, 1199, 1262, 1346, 1379 and 1675 were found in the pubMLST database for isolates from the east and west coast of the North America, Chile, Europe and Southeast Asia (Figure 21).

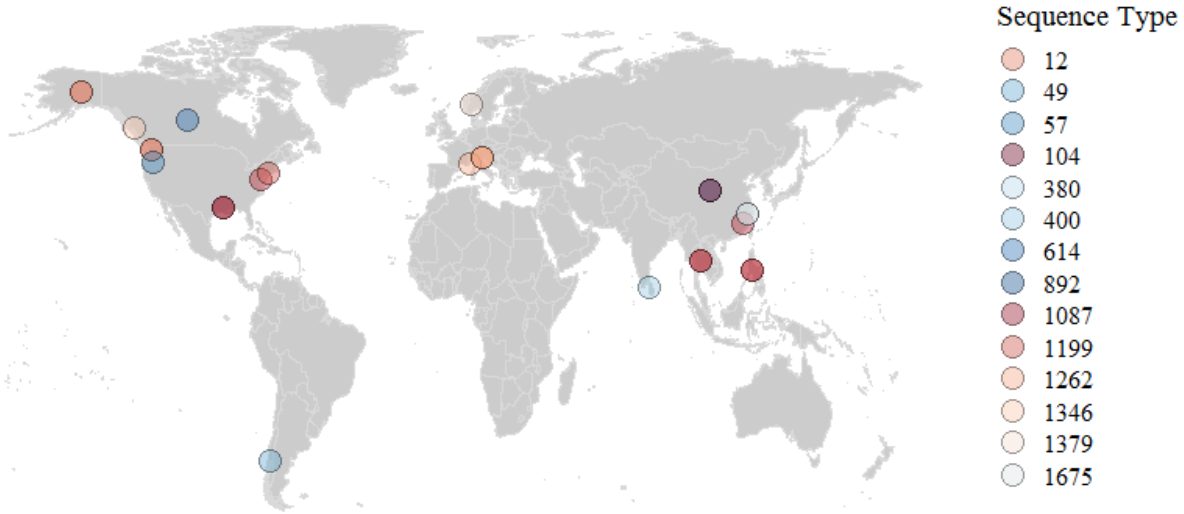


Figure 21. Fifteen ST identified in GBE isolates were reported from clinical and environmental isolates across North America, South America, Europe and South East Asia.

ST 614, 892 and 1675 were previously recovered as clinical isolates in Canada and China, whereas multiple ST 12 isolates from Alaska were previously *trh*+ and Urease+. Seven isolates from the SRA and six GBE isolates from ST 12, 49 and 114 shared 3,930 core genes and 4,303 genes were variable. The extra GBE core gene content was also present in the ST core genome and no variable gene content was significantly unique to GBE isolates. This is consistent with outcomes from section 3.1.3 that determined that the strongest predictor of variable genome content was lineage, though some differences in isolates from the GBE within ST were observed (Figure 22).

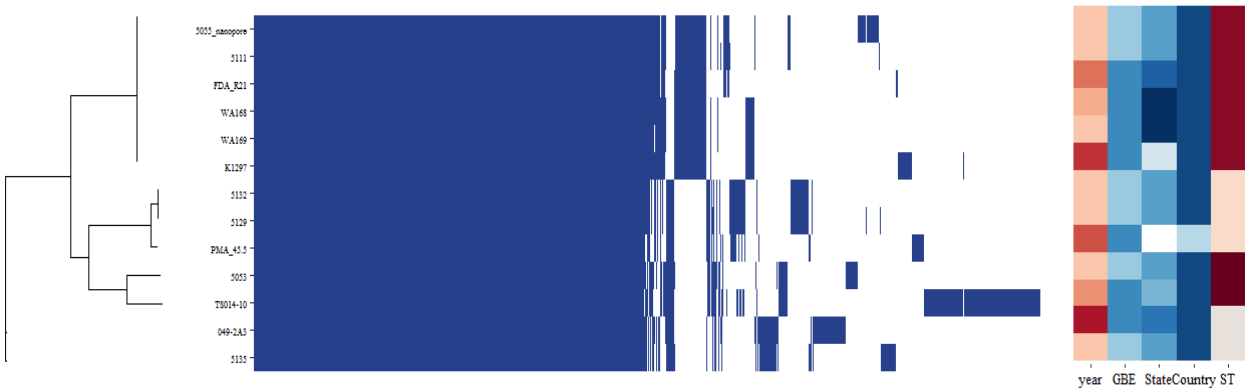


Figure 22. Pangenome analysis with maximum-likelihood tree of the core genes from ST 12, 49, 57 and 114 isolated from the GBE and assembled from the SRA. Blue represents the presence of genes whereas white space is the absence of genes. Gene organization is determined by the frequency of each gene in the pangenome. The color bar is ordered as year, GBE, State, Country and ST.

For example, 327 variable genes are conserved within the ST 12 (Figure 22, colorbar #6, medium red), and are not present in the other ST lineages. Though the majority of this content is annotated as hypothetical proteins (231/327), there are also multiple cold shock proteins, genes related to Type III secretion systems and urease including: *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, *ureG*, *ureR* and *tdh2*. ST 12 from Washington and Alaska shared 97 genes that were not found in ST 12 isolates from the GBE. Though 67/97 of these genes were annotated as hypothetical proteins, three copies of genes related to prophage integrase and anti-toxin *hipB* were present. Similarly, GBE ST 12 isolates 5055 and 5111 shared 33 unique genes, though 26/33 were annotated as hypothetical proteins, 2 additional *recA* genes were identified. Isolate 5055 is also notable because of the presence of a plasmid that is not found in other isolates from ST 12. There were 79 mostly hypothetical proteins that were present in ST 49, 57 and 114 that were not present in ST 12.

Though ST 12 was differentiated from other lineages by allelic diversity in the core genome, the majority (n= 77) of the 97 unique genes specific to ST 49 were hypothetical proteins. ST 49 isolates 5129 and 5132 from the GBE contained 180 additional genes that were not present in the ST 49 isolate from Chile. This variable content notably included multiple cold shock genes, DNA polymerase, helicase, recombinase and prophage integrase, however the majority (160/197) were annotated as hypothetical protein.

There was a striking difference in variable content in ST 114, where the previous isolate was recovered from sediment Maryland in 2010. 1095/1272 genes were hypothetical proteins, though genes that were annotated for function included multiple genes related to plasmid partition proteins, prophage integrase, anaerobic sulfatase and thiol: disulfide interchange. Though 80 unique, mostly hypothetical proteins were shared within ST 114, the large stretch of genes in isolate TS014-10 were not present in ST 114 isolate 5053 in the GBE. ST 57 also contained variable genetic content that was unique to the lineage as well as the isolate from the GBE, though more than 85% of these genes were annotated as hypothetical proteins. The annotated genes within isolate 5135 from the GBE included functions for prophage integrase, transposon resolvase, DNA polymerase and recombinase. Therefore, though the allelic variation in the core genome and the majority of variable content appears to be largely lineage specific in this study, there are also clear differences within lineages that may be driven by lifestyle as well.

3.3 Genome wide association study

The core and accessory genes of the *Vibrio parahaemolyticus* pangenome were also assessed for patterns of allelic diversity, gene content and function related to their relative ST, date, site or matrix of collection. The conservation of the genes in the core genome was highly variable. There were 313,326 SNPs in genes of the core genome and 25,545 multi-allelic sites. Some genes were highly conserved, with fewer than 10 SNPs found in forty genes in the core genome and no SNPs found in five genes in the core genome, including: *acpP*, *rplN*, *rplR*, *rpmD*, *rpsH*, *rpsT* and *rpsU*. There were approximately 49,796 ±7,681 SNPs in each isolate. Pairwise comparison did not find a statistically significant difference between the allelic diversity of genes in the core genome relative to the metadata. Though, multivariate analysis with ISA suggested that the variation in

a handful of core genes SNPs may be uniquely associated with source or date (Table 18), phylogenetic clustering with maximum likelihood was most consistent with ST (Fig. 22).

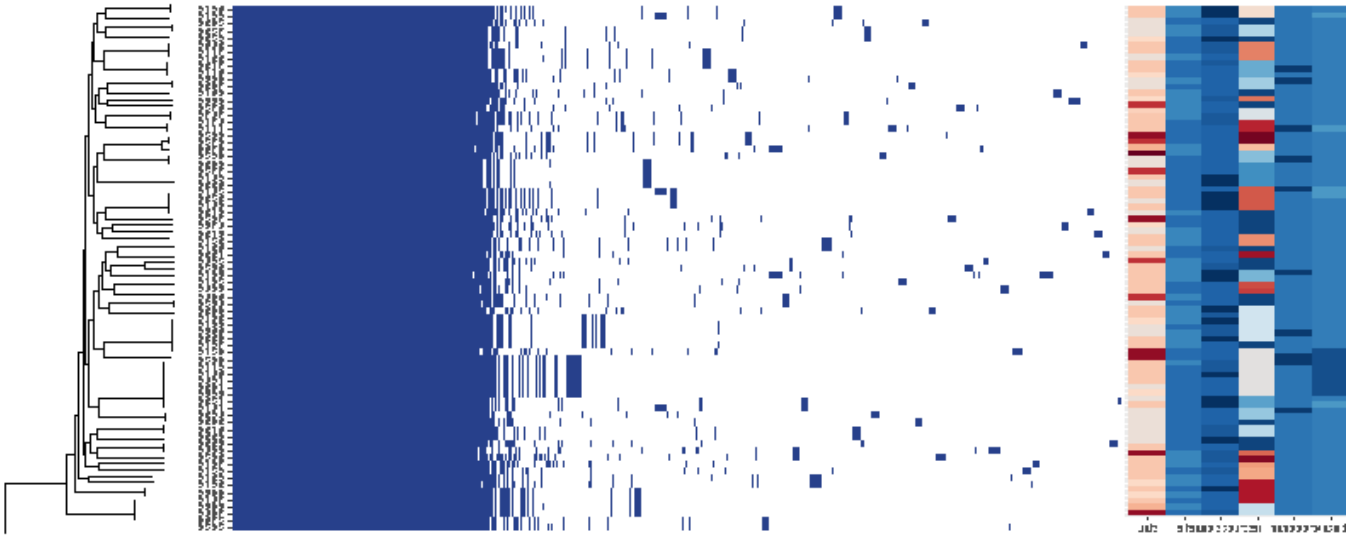


Figure 23. Pangenome analysis with maximum-likelihood phylogenetic tree of 3,931 core genes. Blue represents the presence of genes whereas white space is the absence of genes. Gene organization is determined by the frequency of each gene in the pangenome. The color bar is ordered as date, site, matrix, ST, nanopore and plasmid (dark blue = small plasmid, light blue = large plasmid)

Table 18. MRPP and ISA of the allelic diversity in the core genome of the variable genes and function in the accessory genome for ST, date, site and matrix.

Category	Core Allelic		Variable Genes		Variable Function	
	p	n	p	n	p	n
Sequence Type	0.41	In progress	0.076	In progress	0.3	In progress
Date	0.71	10	0.07	603	0.67	3
Site	0.62	0	0.309	62	0.64	1
Matrix	0.70	4	0.069	443	0.50	3

The most apparent grouping between isolates was by ST (figure 23, color bar #4) and qualitatively, the variable content within each ST lineage appears to be highly clonal by presence/absence. This was confirmed by pairwise and multivariate analysis of the variable genes and function in the accessory genome for ST, date, site and matrix (Table 18). The lack of

significance of the MRPP and unique genes identified by ISA for date, matrix and ST requires further investigation to account for the complex combination of overlap between the three variables. For example, ST A appears to be largely clonal and it is the only group with the small plasmid in this collection of isolates. The plasmid that is accessory content of the pangenome may be specific to ST A (Figure 8, dark blue color bar #6), though isolates from ST A and isolates with the large plasmid were collected throughout the study from multiple combinations of dates, sites and matrix. The large plasmid (Figure 8, light blue color bar #6) that was identified in ST 12 and 1379 is present in multiple distinct lineages. Similarly, *ureB, ureC, ureD, ureE, ureF, ureG, ureR* and *tdh2* were also present in ST 12 isolates 5111 and 5055 on chromosome one. However isolates 5170, 5196, 5707 and 6244. did not have the large plasmid, were from three distinct, previously unreported lineages collected in July, August and September from both sites and all matrices but had *ureB, ureC, ureD, ureE, ureF, ureG, ureR* and *tdh2*. So, statistically robust differences based on site, date or matrix that may suggest ecological relevance of the diversity in gene content will require more controlled, disaggregated comparisons.

4. DISCUSSION

The diversity among clinical and environmental *Vibrio parahaemolyticus* populations has frequently been examined using multilocus sequence analysis and these studies reveal highly diverse populations. Many hypothesize that organizing the diversity of *V. parahaemolyticus* to environmentally relevant structures within these populations could be key to understanding and forecasting *V. parahaemolyticus* dynamics and disease risk (eg: Fraser, 2009). However, assigning this diversity to ecological drivers has proven to be complex (eg: Umersbach et al., 2014; Ellis et al., 2012; Thompson et al., 2004). Ecotypes provide an appealing mechanism to connect environmental conditions to population dynamics and the water column provides a diverse range of resources and nutrient patchiness that is an ideal environment for the identification of micro-niche specialized ecotypes (Shapiro and Polz, 2014, Cordero and Polz, 2014, Hunt *et al.*, 2008, Johnson *et al.*, 2006; Cohan *et al.*, 2001), though, overall, ecotypes have not been frequently identified within environmental populations (Bendell *et al.*, 2016).

This process of evaluating the genetic diversity of a population to better characterize its ecology is known as a reverse ecology framework. It integrates characteristics of genetic diversity including SNP profiles, core and accessory genetic content and recombination rates to provide insight into the forces creating and maintaining genomic diversity within a local population. *Vibrio parahaemolyticus* may also more rapidly undergo environmental adaptation through its smaller, second chromosome. The second chromosome in *Vibrio* is hypothesized to be the result of an acquired plasmid that provided a fitness benefit and was maintained. Previous studies have frequently noted that the second chromosome is enriched in genetic function related to environmental persistence supporting the theory that the second chromosome specializes in functions related to environmental adaptation and persistence (Ellis et al., 2012, Makino et al., 2003, Morrow and Cooper, 2012). Together, these analysis of these traits can provide an enhanced picture of the ecology of the *V. parahaemolyticus*.

In this study, chromosome one contained on average 2,939 ± 50 coding sequences and chromosome two had approximately 1,558 ± 28 coding sequences. Genes in the core and accessory genome were present on both the first and second chromosome and the overall the gene function in the core genome at the highest level of the acyclic GO hierarchical structuring was significantly enriched in functions related to metabolic processes. There were differences in enriched function between core genes on the first and second chromosome. For example, cellular metabolic process, primary metabolic process and nitrogen compound metabolic process, were significantly enriched in the core genes on the first chromosome, whereas localization, biological regulation, signaling and transport were significantly enriched in the core content on the second chromosome. The genes in the accessory genome were enriched for function related to DNA recombination, and transposition as well as defense response, viral process and assembly, symbiont response. Though 1,677 genes were part of the GBE core compared to the 2,254 gene cgMLST suggested by Gonzalez-Escalona et al., (2017), both the cgMLST and extra GBE core genes mapped equally to the first or second chromosome. The functional differences suggest that the cgMLST may be enriched for metabolism, whereas the extra GBE core was enriched for localization, cell projection organization, transport and signaling. However, further work is needed to assess the biological validity of these differences, especially in the context of differential

chromosomal functional enrichment due to the relatively low number of genes assigned with GO terms.

The relevance of these differences was assessed in light of the cgMLST because the assignment of genes as core and accessary may largely dependent on the set of isolates within the collection being analyzed. So, genes that are considered part of the core genome in this study may in fact be accessory content in a larger or more diverse study. In the collection of isolates representing ST 12, 49, 57, 114, from the GBE and outside the GBE, the extra GBE core gene content was also present in the ST core genome (though the BLASTP identity in this analysis was set to 95 compared to 90 for the GBE run, so the ST size of the core genome will likely increase once this is corrected). It's clear that the GBE core in this study largely relates to the ST composition of the isolates. This highlights that a cgMLST for *Vibrio parahaemolyticus* could provide the basis for more a more robust and standardized evaluation of the accessory genome across studies by controlling for the potential bias of assigning genes as core or accessory based on sampling bias.

In both the ST and GBE pangenome study, lineage was the strongest predictor of isolate clustering based on core genome variation. The variable content within ST groups was largely conserved within lineages, though it was clear that isolates within each ST had acquired new genetic content that was not present in other isolates from the same lineage or location. For example, ST 12 contained multiple cold shock proteins, genes related to Type III secretion systems and ureases including: *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, *ureG*, *ureR*, as well as *tdh2*. However, these genes were also found in four isolates from previously unidentified ST in the GBE. ST 12 isolate 5055 was also notable because of the presence of a plasmid that was not found in other isolates from ST 12, including GBE 5111. The ST 114 isolate recovered from sediment in Maryland in 2010 contained an additional 1272 genes compared to GBE ST 114 isolate 5053. Likewise, the overall difference of the variable genes and function in the accessory genome for ST, date, site and matrix was largely attributed to lineage. However, the ISA for date, matrix and ST suggest differences in genomic content related to the genes that are present in the individual accessory genomes of isolates from those groups. Though differences based on site, date or matrix may suggest ecological relevance

of the diversity in gene content, this will require more controlled, disaggregated comparisons to detect lifestyle driven differences within the overall influence of lineage.

The structure and content of the *Vibrio parahaemolyticus* genome is largely facilitated by variable mutation rates coupled with combined with gene gain and loss. The overall content consists predominantly of coding sequences (identified and hypothetical) with very little intragenic or pseudogenic content (~5,000 orf and 5mb). Homologous recombination also provides the basis for niche adaptation and diversification, for example the current genetic divergence within ST36 clonal population has been attributed to recombination (Martinez-Urtuza et al., 2017). Whereas the most recognized mechanism for rapid niche adaptation in *V. parahaemolyticus* is homologous recombination and horizontal gene transfer, Johnson et al., (2013) suggested that elevated mutation rates may also facilitate niche adaptation. A current limitation of this study is the emphasis on gene presence/absence and currently work is in process to more robustly assess the allelic diversity of the pangenomes in this study, including analysis of recombination, selection and linkage disequilibrium. This will also enable consideration of the majority of variable content that is hypothetical proteins and not functionally annotated. Another potential direction that needs to be considered is gene content that is orthologous at levels below 90% identity.

We show that the GBE *Vibrio parahaemolyticus* genomes are shaped by both lineage and the environment, similar to findings by Tamames et al., (2016) and there is evidence of strains from globally disparate regions remaining well-conserved and persisting over time. Though variable content and allelic diversity are largely based on lineage in the GBE, there is also evidence of the acquisition of novel genetic material that is integrated into the chromosome or present on plasmids that are shared between distinct lineages consistent with findings by Bobay and Ochman, (2017), Ceccarelli et al., (2013), Johnson, (2013) and Metzger and Blokesch, (2014).

The diversity within and between *Vibrio parahaemolyticus* populations has been proposed to arise from the demands of the environment they inhabit. This idea of "niche sequestering" was observed and reported by Johnson et al., (2012) in strains from the Gulf of Mexico and Shapiro and Polz (2014) where *V. parahaemolyticus* strains from similar environmental niches were more similar in genetic content and allelic diversity than they were to strains that were isolated from

different environmental conditions. Whereas both variable mutation rates across the genome and horizontal gene transfer and have been shown to play a role in the evolution of *V. parahaemolyticus* (Tamames et al., 2016), many believe that HGT and recombination is the predominant force shaping the *V. parahaemolyticus* genome (Martinez-Urtaza et al., 2017; Ceccarelli et al., 2013; Johnson et al., 2013).

Historically, phylogenetic assessment has been the most widely used epidemiological tool to trace the evolutionary background or trajectory of the emergence of virulence within a population, however, the ability of *Vibrio parahaemolyticus* to undergo recombination, even in conserved regions believed to be limited to vertical acquisition, potentially masks many of the patterns typically used to assess the development and succession of virulent lineages within largely non-pathogenic populations. Given this, effective epidemiological reconstruction and investigation of the mechanisms of pathogen evolution will require accounting for the many potential sources of underlying diversity within populations. Environmentally relevant structure within these populations could be key to understanding the diversity and drivers underlying community dynamics and cycling in the estuarine environment. The relationship between genetic diversity and the environment in the GBE remains to be clarified, however ecotype differentiation is an important potential direction to better determining the basis for how environmental adaptation shapes *V. parahaemolyticus* dynamics

References

Alexa A, Rahnenfuhrer J (2019). *topGO: Enrichment Analysis for Gene Ontology*. R package version 2.36.0.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410

Andrew J. Page, Carla A. Cummins, Martin Hunt, Vanessa K. Wong, Sandra Reuter, Matthew T. G. Holden, Maria Fookes, Daniel Falush, Jacqueline A. Keane, Julian Parkhill, Bioinformatics, (2015). "Roary: Rapid large-scale prokaryote pan genome analysis", doi: http://dx.doi.org/10.1093/bioinformatics/btv421

Andrews, S. (2010). FASTQC. A quality control tool for high throughput sequence data

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., & Martinez-Urtaza, J. (2017). Non-Cholera *Vibrios*: The Microbial Barometer of Climate Change. Trends in Microbiology. https://doi.org/10.1016/j.tim.2016.09.008

Bendall, M. L., Stevens, S. L. R., Chan, L. K., Malfatti, S., Schwientek, P., Tremblay, J., Malmstrom, R. R. (2016). Genome-wide selective sweeps and gene-specific sweeps in natural bacterial populations. *ISME Journal*, *10*(7), 1589–1601. https://doi.org/10.1038/ismej.2015.241

Blackwell, K. D., & Oliver, J. D. (2008). The ecology of *Vibrio vulnificus, Vibrio cholerae,* and *Vibrio parahaemolyticus* in North Carolina estuaries. *Journal of Microbiology (Seoul, Korea)*, *46*(2), 146–153. https://doi.org/10.1007/s12275-007-0216-2

Bobay, L.-M., & Ochman, H. (2017). Biological Species Are Universal across Life's Domains. *Genome Biology and Evolution*, *9*(3), 491–501. https://doi.org/10.1093/gbe/evx026

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114-2120.

Brynildsrud O, Bohlin J, Scheffer L, Eldholm V. Rapid scoring of genes in microbial pan-genome-wide association studies with Scoary. *Genome Biol*. 2016;17:238

Carlson M (2019). *GO.db: A set of annotation maps describing the entire Gene Ontology*. R package version 3.8.2

Ceccarelli, D., Hasan, N. A., Huq, A., & Colwell, R. R. (2013). Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Frontiers in Cellular and Infection Microbiology*, 3. https://doi.org/10.3389/fcimb.2013.00097

Centers for Disease Control and Prevention (CDC). (2013). Increase in *Vibrio parahaemolyticus* illnesses associated with consumption of shellfish from several Atlantic coast harvest areas, United States, 2013. *Vibrio* Illness (Vibriosis), 21.

Cohan, F. M. (2002). What are Bacterial Species? *Annual Review of Microbiology*, *56*(1), 457–487.

https://doi.org/10.1146/annurev.micro.56.012302.160634

Cooper, V. S., Vohr, S. H., Wrocklage, S. C., & Hatcher, P. J. (2010). Why genes evolve faster on secondary chromosomes in bacteria. *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1000732

Coordinators, N. R. (2016). Database resources of the national center for biotechnology information. Nucleic acids research, 44(Database issue), D7.

Cordero, O. X., & Polz, M. F. (2014). Explaining microbial genomic diversity in light of evolutionary ecology. *Nature Reviews Microbiology*. Nature Publishing Group. https://doi.org/10.1038/nrmicro3218

Cui, Y., Yang, X., Didelot, X., Guo, C., Li, D., Yan, Y., Zhang, Y., Yuan, Y., Yang, H., Wang, J. and Wang, J., 2015. Epidemic clones, oceanic gene pools, and eco-LD in the free living marine pathogen *Vibrio parahaemolyticus*. *Molecular biology and evolution*, *32*(6), pp.1396-1410.

Danecek P, Auton A, Abecasis G, Albers CA, Banks E et al.Bioinformatics (Oxford, England) 2011;27;15;2156-8PUBMED: 21653522; PMC: 3137218; DOI: 10.1093/bioinformatics/btr33

Davey, M. E., & O'toole, G. A. (2000). Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/MMBR.64.4.847-867.2000

Davis, B. J., Jacobs, J. M., Davis, M. F., Schwab, K. J., DePaola, A., & Curriero, F. C. (2017). Environmental determinants of Vibrio parahaemolyticus in the Chesapeake Bay. Appl. Environ. Microbiol., 83(21), e01147-17.

Davis, B. J., Jacobs, J. M., Zaitchik, B., DePaola, A., & Curriero, F. C. (2019). *Vibrio parahaemolyticus* in the Chesapeake Bay: operational in situ prediction and forecast models can benefit from inclusion of lagged water quality measurements. *Applied and environmental microbiology*, 85(17), e01007-19.

De Caceres, M., Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. Ecology, URL http://sites.google.com/site/miqueldecaceres/

Deter, J., Solen, L., Antoine, V., Jaufrey, J., Annick, D., Dominique, H.H. (2010). Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. Environmental microbiology, 12(4), 929-937.

Deepanjali, A., Kumar, H., Karunasagar, I., Karunasagar, I. Seasonal Variation in Abundance of Total and Pathogenic *Vibrio parahaemolyticus* in Oysters along the Southwest Coast of India. *Applied and Environmental Microbiology*. (2005) 71, 7. http://aem.asm.org/content/71/7/3575.abstract

DePaola, A., Ulaszek, J., Kaysner, C. A., Tenge, B. J., Nordstrom, J. L., Wells, J., Gendel, S. M. (2003). Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. *Applied*

and Environmental Microbiology. https://doi.org/10.1128/AEM.69.7.3999-4005.2003

DePaola, A.; Hopkins, L.H.; Peeler, J.T.; Wentz, B.; McPhearson, R.M. Incidence of *Vibrio parahaemolyticus* in US coastal waters and oysters. *Appl. Environ. Microbiol*. 1990, 1;56(8), 2299-302.

Ellis, C. N., Schuster, B. M., Striplin, M. J., Jones, S. H., Whistler, C. A., & Cooper, V. S. (2012). Influence of seasonality on the genetic diversity of *Vibrio parahaemolyticus* in New Hampshire shellfish waters as determined by multilocus sequence analysis. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.07794-11

Espejo, R. T., García, K., & Plaza, N. (2017, July 24). Insight into the origin and evolution of the *Vibrio parahaemolyticus* pandemic strain. *Frontiers in Microbiology*. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2017.01397

Emms, D. M., & Kelly, S. (2015). OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome biology, 16(1), 157.

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G., & Hanage, W. P. (2009). The Bacterial Species Challenge : Ecological Diversity. *Science*, *323*(February), 741–746. https://doi.org/10.1126/science.1159388

Friedman, J., Alm, E. J., & Shapiro, B. J. (2013). Sympatric Speciation: When Is It Possible in Bacteria? *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0053539

Froelich, B., Bowen, J., Gonzalez, R., Snedeker, A., & Noble, R. (2013). Mechanistic and statistical models of total *Vibrio* abundance in the neuse river estuary. *Water Research*. https://doi.org/10.1016/j.watres.2013.06.050

Froelich, B. A., & Noble, R. T. (2016). *Vibrio* bacteria in raw oysters: managing risks to human health. Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1689), 20150209.

Fujino, T., Okuno, Y., Nakada, D., Aoyama, A., Fukai, K., Mukai, T., & Ueho, T. (1953). On the bacteriological examination of shirasu-food poisoning. *Medical Journal of Osaka University*, 4(2/3), 299-304.

Gilbert, J. A., Steele, J. A., Caporaso, J. G., Steinbrück, L., Reeder, J., Temperton, B., Field, D. (2012). Defining seasonal marine microbial community dynamics. *The ISME Journal*. https://doi.org/10.1038/ismej.2011.107

Gonzalez-Escalona, N., Jolley, K. A., Reed, E., & Martinez-Urtaza, J. (2017). Defining a core genome multilocus sequence typing scheme for the global epidemiology of *Vibrio parahaemolyticus*. *Journal of clinical microbiology*, *55*(6), 1682-1697.

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. Bioinformatics, 29(8), 1072-1075.

Hartwick, M., Berenson, A., Whistler, C.A., Cooper, V.S., and Jones, S.H. The Seasonal Ecology of *Vibrio parahaemolyticus* and Plankton in a New Hampshire Estuary (in preparation)

Hartwick, M., Urquhart, A.E., Whistler, C.A., Cooper, V.S., Naumova, E.N., and Jones, S.H. Forecasting *Vibrio parahaemolyticus* Concentration in Shellfish from a New England Estuary (submitted)

Holden, M. T. G., Titball, R. W., Peacock, S. J., Cerdeño-Tárraga, A. M., Atkins, T., Crossman, L. C., Parkhill, J. (2004). Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(39), 14240–14245. https://doi.org/10.1073/pnas.0403302101

Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J., & Polz, M. F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science*, *320*(5879), 1081–1085. https://doi.org/10.1126/science.1157890

Inouye, M., Dashnow, H., Raven, L.A., Schultz, M.B., Pope, B.J., Tomita, T., Zobel, J. and Holt, K.E., 2014. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. Genome medicine, 6(11), p.90.

Johnson, C. N. (2013). Fitness Factors in Vibrios: A Mini-review. *Microbial Ecology*, *65*(4), 826–851. https://doi.org/10.1007/s00248-012-0168-x

Jones, S.; Summer-Brason, B. Incidence and detection of pathogenic *Vibrio* spp. in a northern New England estuary, USA. Journal of Shellfish Research 1998, 17, 1665–1669.

Kaneko and, T., & Colwell2, R. R. (1973). Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. *JOURNAL OF BACTERIOLOGY*, *113*(1), 24–32.

Kaneko, T., & Colwell, R. R. (1975). Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Environ. Microbiol., 30(2), 251-257.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution*, 30(4), 772-780

Keymer, D. P., Miller, M. C., Schoolnik, G. K., & Boehm, A. B. (2007). Genomic and phenotypic diversity of coastal *Vibrio cholerae* strains is linked to environmental factors. *Appl. Environ. Microbiol.*, 73(11), 3705-3714.

Leinonen, R., Sugawara, H., Shumway, M., & International Nucleotide Sequence Database Collaboration. (2010). The sequence read archive. Nucleic acids research, 39(suppl_1), D19-D21.

Le Roux, F., & Blokesch, M. (2018). Eco-evolutionary dynamics linked to horizontal gene transfer in vibrios. *Annual review of microbiology*, 72, 89-110.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, and Richard Durbin. The sequence alignment/map format and SAMtools. *Bioinformatics* 25, no. 16 (2009): 2078-2079.

Lopez-Hernandez, K. M., Pardio-Sedas, V. T., Lizarraga-Partida, L., Williams, J. de J., Martinez-Herrera, D., Flores-Primo, A., Rendon-Castro, K. (2015). Environmental parameters influence on the dynamics of total and pathogenic *Vibrio parahaemolyticus* densities in *Crassostrea virginica* harvested from Mexico's Gulf coast. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2014.11.015

Lovell, C. R. (2017). Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-017-8096-9

Löytynoja, A. (2014). Phylogeny-aware alignment with PRANK. In Multiple sequence alignment methods (pp. 155-170). Humana Press, Totowa, NJ.

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iida, T. (2003). Genome sequence of *Vibrio parahaemolyticus*: A pathogenic mechanism distinct from that of *V. cholerae*. *Lancet*. https://doi.org/10.1016/S0140-6736(03)12659-1

Martinez-Urtaza, J., Baker-Austin, C., Jones, J. L., Newton, A. E., Gonzalez-Aviles, G. D., & DePaola, A. (2013). Spread of Pacific Northwest *Vibrio parahaemolyticus* Strain. *New England Journal of Medicine*. https://doi.org/10.1056/NEJMc1305535

Martinez-Urtaza, J., Blanco-Abad, V., Rodriguez-Castro, A., Ansede-Bermejo, J., Miranda, A., & Rodriguez-Alvarez, M. X. (2012). Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus* in offshore areas. *The ISME Journal*. https://doi.org/10.1038/ismej.2011.156

Martinez-Urtaza, J., Lozano-Leon, A., Varela-Pet, J., Trinanes, J., Pazos, Y., & Garcia-Martin, O. (2008). Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the rias of Galicia, Spain. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.01307-07

Martinez-Urtaza, J., Van Aerle, R., Abanto, M., Haendiges, J., Myers, R. A., Trinanes, J., Gonzalez-Escalona, N. (2017). Genomic variation and evolution of *Vibrio parahaemolyticus* ST36 over the course of a transcontinental epidemic expansion. *MBio*, *8*(6). https://doi.org/10.1128/mBio.01425-17

Meibom, K. L., Blokesch, M., Dolganov, N. A., Wu, C. Y., & Schoolnik, G. K. (2005). Microbiology: Chitin induces natural competence in *Vibrio cholerae*. *Science*, *310*(5755), 1824–1827. https://doi.org/10.1126/science.1120096

Metzger, L. C., & Blokesch, M. (2014). Composition of the DNA-uptake complex of *Vibrio cholerae*. *Mobile Genetic Elements*, *4*(1), e28142. https://doi.org/10.4161/mge.28142

Morrow, J. D., & Cooper, V. S. (2012). Evolutionary effects of translocations in bacterial genomes. *Genome Biology and Evolution*. https://doi.org/10.1093/gbe/evs099

Nilsson, W. B., Paranjpye, R. N., Hamel, O. S., Hard, C., & Strom, M. S. (2019). *Vibrio parahaemolyticus* risk assessment in the Pacific Northwest: it's not what's in the water.

FEMS Microbiology Ecology, *95*(4). https://doi.org/10.1093/femsec/fiz027

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelsky, A., Pyshkin, A., Sirotkin, A., Sirotkin, Y. and Stepanauskas, R., 2013, April. Assembling genomes and mini-metagenomes from highly chimeric reads. In Annual International Conference on Research in Computational Molecular Biology (pp. 158-170). Springer, Berlin, Heidelberg.

Oberbeckmann, S., Wichels, A., Wiltshire, K. H., & Gerdts, G. (2011). Occurrence of Vibrio parahaemolyticus and Vibrio alginolyticus in the German Bight over a seasonal cycle. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. https://doi.org/10.1007/s10482-011-9586-x

Oberbeckmann, S., Fuchs, B. M., Meiners, M., Wichels, A., Wiltshire, K. H., & Gerdts, G. (2012). Seasonal dynamics and modeling of a *Vibrio* community in coastal waters of the North Sea. Microbial ecology, 63(3), 543-551.

Page, A.J., Taylor, B., Delaney, A.J., Soares, J., Seemann, T., Keane, J.A., Harris, S.R.,SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments", Microbial Genomics 2(4), (2016)

Panicker, G.; Call, D. R.; Krug, M. J.; Bej, A. K. Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. Applied and Environmental Microbiology 2004, 70(12), 7436–44.

Paranjpye, R., Hamel, O. S., Stojanovski, A., & Liermann, M. (2012). Genetic diversity of clinical and environmental *Vibrio parahaemolyticus* strains from the Pacific northwest. *Applied and Environmental Microbiology*, *78*(24), 8631–8638. https://doi.org/10.1128/AEM.01531-12

Paranjpye, R. N., Nilsson, W. B., Liermann, M., Hilborn, E. D., George, B. J., Li, Q., Sandifer, P. A. (2015). Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA. *FEMS Microbiology Ecology*. https://doi.org/10.1093/femsec/fiv121

Parveen, S., Hettiarachchi, K. A., Bowers, J. C., Jones, J. L., Tamplin, M. L., McKay, R., DePaola, A. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology*, *128*(2), 354–361. https://doi.org/10.1016/j.ijfoodmicro.2008.09.019

Pedersen TL (2016). PanVizGenerator: Generate PanViz visualisations from your pangenome. R package version 1.2.0, https://github.com/thomasp85/PanVizGenerator.

Pruzzo, C., Vezzulli, L., & Colwell, R. R. (2008, June). Global impact of *Vibrio cholerae* interactions with chitin. *Environmental Microbiology*. https://doi.org/10.1111/j.1462-2920.2007.01559.x

Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio parahaemolyticus* in Raw Oysters | FDA. (n.d.). Retrieved October 29, 2019, from

https://www.fda.gov/food/cfsan-risk-safety-assessments/quantitative-risk-assessment-public-health-impact-pathogenic-vibrio-parahaemolyticus-raw-oysters

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Semenza, J. C., Trinanes, J., Lohr, W., Sudre, B., Löfdahl, M., Martinez-Urtaza, J., Rocklöv, J. (2017). Environmental suitability of *Vibrio* infections in a warming climate: An early warning system. *Environmental Health Perspectives*, *125*(10). https://doi.org/10.1289/EHP2198

Shapiro, B. J., & Polz, M. F. (2014). Ordering microbial diversity into ecologically and genetically cohesive units. *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2014.02.006

Shinoda, S., & Miyoshi, S. ichi. (2006). Hemolysins of *Vibrio cholerae* and other *Vibrio* species. In *The Comprehensive Sourcebook of Bacterial Protein Toxins* (pp. 748–762). Elsevier Inc. https://doi.org/10.1016/B978-012088445-2/50049-4

Sun, F., Zhang, Y., Wang, L., Yan, X., Tan, Y., Guo, Z., Zhou, D. (2012). Molecular Characterization of Direct Target Genes and cis-Acting Consensus Recognized by Quorum-Sensing Regulator *AphA* in *Vibrio parahaemolyticus*. *PLoS ONE*, *7*(9). https://doi.org/10.1371/journal.pone.0044210

Takemura, A. F., Chien, D. M., & Polz, M. F. (2014). Associations and dynamics of *Vibrionaceae* in the environment, from the genus to the population level. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2014.00038

Tamames, J., Sánchez, P. D., Nikel, P. I., & Pedrós-Alió, C. (2016). Quantifying the relative importance of phylogeny and environmental preferences as drivers of gene content in prokaryotic microorganisms. *Frontiers in Microbiology*, *7*(MAR). https://doi.org/10.3389/fmicb.2016.00433

Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., Lim, E., & Polz, M. F. (2004). Diversity and dynamics of a North Atlantic coastal *Vibrio* community. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.70.7.4103-4110.2004

Tonkin, J. D., Bogan, M. T., Bonada, N., Rios-Touma, B., & Lytle, D. A. (2017). Seasonality and predictability shape temporal species diversity. *Ecology*, *98*(5), 1201–1216. https://doi.org/10.1002/ecy.1761

Turner, J. W., Berthiaume, C. T., Morales, R., Armbrust, E. V., & Strom, M. S. (2016). Genomic evidence of adaptive evolution in emergent *Vibrio parahaemolyticus* ecotypes. *Elementa: Science of the Anthropocene*. https://doi.org/10.12952/journal.elementa.000117

Turner, J. W., Malayil, L., Guadagnoli, D., Cole, D., & Lipp, E. K. (2014). Detection of *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae* with respect to seasonal fluctuations in temperature and plankton abundance. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12246

The UniProt Consortium. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Res.

47: D506-515 (2019)

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics, 30(14), 2068-2069.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, 30(9), 1312-1313.

Urmersbach, S., Alter, T., Koralage, M. S. G., Sperling, L., Gerdts, G., Messelhäusser, U., & Huehn, S. (2014). Population analysis of *Vibrio parahaemolyticus* originating from different geographical regions demonstrates a high genetic diversity. *BMC Microbiology*, *14*, 59. https://doi.org/10.1186/1471-2180-14-59

Urquhart, E. A., Jones, S. H., Yu, J. W., Schuster, B. M., Marcinkiewicz, A. L., Whistler, C. A., & Cooper, V. S. (n.d.). Environmental Conditions Associated with Elevated *Vibrio parahaemolyticus* Concentrations in Great Bay Estuary, New Hampshire. https://doi.org/10.1371/journal.pone.0155018

Venkateswaran, K., Kiiyukia, C., Nakanishi, K., Nakano, H., Matsuda, O., & Hashimoto, H. (1990). The role of sinking particles in the overwintering process of *Vibrio parahaemolyticus* in a marine environment. FEMS Microbiology Ecology, 6(2), 159-166.

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P. C., Colwell, R. R., Höfle, M. G., & Pruzzo, C. (2012). Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. *The ISME Journal*. https://doi.org/10.1038/ismej.2011.89

Vezzulli, L., Pezzati, E., Moreno, M., Fabiano, M., Pane, L., Pruzzo, C., & The VibrioSea Consortium. (2009). Benthic ecology of *Vibrio* spp. and pathogenic *Vibrio* species in a coastal Mediterranean environment (La Spezia Gulf, Italy). *Microbial Ecology*. https://doi.org/10.1007/s00248-009-9542-8

Weinert, L. A., & Welch, J. J. (2017, December 1). Why Might Bacterial Pathogens Have Small Genomes? *Trends in Ecology and Evolution*. Elsevier Ltd. https://doi.org/10.1016/j.tree.2017.09.006

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS computational biology, 13(6), e1005595.

Wick, R. R., Schultz, M. B., Zobel, J., & Holt, K. E. (2015). Bandage: interactive visualization of de novo genome assemblies. Bioinformatics, 31(20), 3350-3352.

Williams, T. C., Ayrapetyan, M., & Oliver, J. D. (2014). Implications of chitin attachment for the environmental persistence and clinical nature of the human pathogen *Vibrio vulnificus. Appl. Environ. Microbiol*., 80(5), 1580-1587.

Xu, F., Gonzalez-Escalona, N., Haendiges, J., Myers, R. A., Ferguson, J., Stiles, T., Whistler, C. A. (2017). Sequence type 631 *Vibrio parahaemolyticus*, an emerging foodborne pathogen in North America. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.02162-16

Xu, F., Ilyas, S., Hall, J. A., Jones, S. H., Cooper, V. S., & Whistler, C. A. (2015). Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2015.00272

Yang, C., Pei, X., Wu, Y., Yan, L., Yan, Y., Song, Y., Coyle, N.M., Martinez-Urtaza, J., Quince, C., Hu, Q. and Jiang, M., 2019. Recent mixing of *Vibrio parahaemolyticus* populations. *The ISME Journal*, p.1.

Young, I., Gropp, K., Fazil, A., & Smith, B. A. (2015). Knowledge synthesis to support risk assessment of climate change impacts on food and water safety: A case study of the effects of water temperature and salinity on Vibrio parahaemolyticus in raw oysters and harvest waters. Food Research International, 68, 86-93.

Yu G, Smith D, Zhu H, Guan Y, Lam TT (2017). ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods in Ecology and Evolution*, 8, 28-36. doi: 10.1111/2041-210X.12628, http://onlinelibrary.wiley.com/doi/10.1111/2041-210X.12628/abstract

Zimmerman, A. M., DePaola, A., Bowers, J. C., Krantz, J. A., Nordstrom, J. L., Johnson, C. N., & Grimes, D. J. (2007). Variability of total and pathogenic *Vibrio parahaemolyticus* densities in Northern Gulf of Mexico water and oysters. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.01700-07